

Investigation into Adverse Reactions to Mycoprotein

Alan George Shand
MBChB (Commend), MRCP(UK)

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Dedication and Acknowledgements

I dedicate this work to Professor Anne Ferguson, late Professor of Gastroenterology, who originally supervised this project but tragically died before its completion.

I am indebted to Dr Marian Aldhous, who performed the preparatory work necessary to develop ELISA assays for mycoprotein and egg albumin, performed the competition ELISAs and patiently oversaw the experimental work described within this thesis.

I would like to express my grateful thanks to Marlow Foods Ltd. who commissioned and sponsored the project, and to Dr Subrata Ghosh, who continued to support and supervise me, after Anne's death.

I thank all the individuals who took the time to reply to my questionnaire, especially those who agreed to send a sample of blood and who travelled to Edinburgh for other tests.

Finally, I thank my dear wife, Nicola, who has cheerfully endured my ever-changing moods throughout the various stages of the project.

Declaration

I, Alan George Shand, declare that this thesis is my own composition. The experimental work contained within this thesis was performed by myself under the expert guidance of Dr Marian Aldhous, except where performed by Dr Aldhous herself, as acknowledged above. All the work contained within this thesis was performed in the Gastrointestinal Laboratory, Western General Hospital, Edinburgh.

The material contained within this thesis formed the basis for a confidential report on the investigation of adverse reactions to mycoprotein, commissioned by Marlow Foods Ltd. No part of the work has been submitted for any other degree or professional qualification.

16/8/02

Abstract

Quorn® is a novel food produced from mycoprotein filaments from the *Fusarium* species ATC20334 (formerly *Fusarium graminearum*) bound together using egg protein. It has been available in the United Kingdom since 1985 and over 15 million packs have been sold in this country.

A small number of individuals have reported adverse reactions following ingestion of this foodstuff including vomiting, skin rashes and even anaphylaxis. There have been no fatalities but reactions are uncomfortable and unpleasant. Possible underlying causes included a true immunologically mediated food allergy to either mycoprotein or egg, a toxic effect of mycotoxins known to be produced by this species, causing delayed gastric emptying and vomiting or a directly irritant effect of the foodstuff on the mucosa of the upper gastrointestinal tract. 140 individuals who had experienced such a reaction were contacted and invited to participate in an investigation into its aetiology. Subjects were invited to complete a postal questionnaire detailing the precise nature of their reaction, past medical and drug histories and asking whether they would be prepared to participate in more invasive investigations. These investigations entailed providing a blood sample for detection of IgE, IgG and IgA antibodies to mycoprotein and egg proteins, and undergoing either upper gastrointestinal endoscopy or isotope solid phase gastric emptying studies pre and post oral challenge with mycoprotein. An in house ELISA assay for IgE, IgG and IgA antibodies was developed.

89/140 replied to the questionnaire and 54 sent a blood sample. 32/54 subjects were female with age range 18-82 years.

Affected subjects had a significantly elevated mean concentration of IgG antibodies to mycoprotein compared with normal controls and these levels approached those seen in controls with intestinal diseases such as Crohn's disease, ulcerative colitis and Coeliac disease.

Three subjects agreed to have endoscopy, all had evidence of minor upper gastrointestinal pathology pre- challenge. There was no difference in either the macroscopic or microscopic appearances of the mucosa post- challenge and in particular, no excess of neutrophils or eosinophils. One subject had isotope gastric emptying studies which were normal at baseline and did not change post- challenge.

There is no evidence of a true food allergy directed against either mycoprotein itself or the egg protein binders used in its manufacture. There is no evidence of any serious immunological abnormality in those affected. However, anti-mycoprotein antibodies produced by subjects have a greater affinity for binding mycoprotein *in vitro* than those produced by disease controls. This is unlikely to explain the reaction but may imply some increased susceptibility in affected subjects. Within the constraints of a small sample size, there was no evidence of any direct irritant or inflammatory reaction on the gastric mucosa when small doses of cooked mycoprotein are ingested. There was however, a high prevalence of minor upper gastrointestinal pathology noted on endoscopy of affected subjects, but this may be a chance finding.

Although sample size was too small to make useful comments, gastric emptying was not abnormal before challenge and was not affected by mycoprotein ingestion.

The underlying aetiology of these reactions remains obscure and at present, the best advice to be given to those affected is to avoid mycoprotein- containing foods.

Chapter 1- Introduction And Aims

1.1.0 Introduction

Ut quod ali cibus est aliis fuit acre venenum.

“What is food to one is to others bitter poison.”

Lucretius (c 95-55BC)

“Food, glorious food” remains one of our basic requirements for life. The earliest societies evolved around the need to produce or procure sufficient food to sustain life. Food surpluses formed the basis for early trade, leading first to prosperity and later to warfare and conquest.

However, even earlier than the first century BC (1), it was recognised that for a few unfortunate souls, seemingly wholesome staple foodstuffs could be a source of discomfort, pain and even, in some cases, death.

In our own times, it is difficult to avoid the almost daily reports of the latest “food-scare” in the mass media. Increasingly, people are concerned that the food we eat, much of which is mass-produced and heavily processed may be to blame for a whole variety of symptoms, from diarrhoea to chronic fatigue. We are shaken to the core that something so integral to our existence may have the potential to make us ill.

Leaving aside the issue of food-borne infection, the idea of food intolerance is an attractive explanation for a myriad of symptoms for many otherwise healthy people.

What evidence exists to support the notion of food-related reactions as a clinical entity?

Coeliac disease is the classical case of an intestinal disease where the exposure of a genetically susceptible individual to a noxious element in food, in this case gluten contained in the storage proteins of cereals such as wheat (α -gliadin), rye (secalins) and barley (hordeins) (2), leads to immunologically mediated damage to the intestinal mucosa. Avoidance of these peptides usually leads to alleviation of the symptoms within two weeks and eventual healing of the intestinal mucosa.

Similarly, there is no doubt that allergies to specific foods and food additives exist. In a small number of immunologically susceptible people, these may manifest as eczema, uncomfortable urticarial rashes, oral-allergy syndrome (3), respiratory symptoms or even as life-threatening anaphylactic reactions. In susceptible infants and children, a variety of dietary components, including casein and whey proteins in cow's milk, may trigger gastrointestinal reactions characterised by diarrhoea and abdominal cramps such as dietary protein-induced proctitis of infancy (4), enteropathy (5), or enterocolitis (6,7). These conditions will respond to prompt withdrawal of the offending protein from the diet.

However, do lesser degrees of food-related disease exist? For example, the irritable bowel syndrome is a common condition which has been estimated to affect up to 5% of males and 13% of females in a stratified randomised urban population in the South West of England (8). Could as yet poorly understood reactions to common foodstuffs be implicated in the pathophysiology of this condition?

It is difficult to quantify the number of individuals who experience adverse reactions, ranging from nausea and abdominal pain to diarrhoea and vomiting, following consumption of specific foods. Such subjective food intolerances are commonplace and may never come to medical attention since they are, generally, simply dealt with by avoidance of the offending substance. It is equally difficult to provide an adequate explanation for such idiosyncratic reactions.

What are the possible mechanisms which underlie such intolerances? Do they have any role to play in triggering or perpetuating the chronic intestinal inflammation seen in individuals with inflammatory bowel diseases such as Crohn's Disease or Ulcerative colitis? If so, could dietary manipulation help to ease the symptoms, or the inflammation associated with these diseases?

1.2.0 Background to adverse reactions to mycoprotein

The fungal species *Fusaria* is widely distributed in nature and can infect a wide variety of plant species. One strain of *Fusarium*, ATCC 20334, previously known as *Fusarium graminearum* has been cultured to produce a novel foodstuff which is marketed in the UK as Quorn[®] (Marlow Foods Ltd, Middlesbrough, UK). This is a high fibre, low fat foodstuff with a protein content similar to whole eggs, a texture which resembles lean meat and is suitable for consumption by vegetarians. A study of the safety, tolerability and nutritional value of this fungal species in 1984 (9) reported no adverse effects in 100 subjects and it was deemed safe for human consumption.

The organism is fermented continuously on a glucose substrate, under strictly controlled conditions to produce large quantities of the basic fungal- or myco-protein which is composed largely of fungal mycelia and chitin. The fibrous fungal mycelia can then be mixed with binders, such as egg protein, then textured and shaped to produce a variety of food products including sausages, burgers and fillets.

Since its introduction in the UK in 1985, over 15 million packs of mycoprotein-containing foodstuffs have been sold. During that same period a small number of individuals have contacted the manufacturer, drawing their attention to reactions which they have suffered in the context of eating these mycoprotein-containing products. A small number of these individuals have suffered symptoms, which are classical of food-borne infection, with vomiting and diarrhoea affecting all those who consumed the meal. This is, of course, unfortunate for the individuals affected, but like most cases of “food poisoning,” may relate more to food handling and preparation practices than to any component of the food itself.

However, the majority of correspondents described symptoms which might only affect one member of the family and which were difficult to ascribe to contamination of the food with enteric pathogens. Indeed, any initial reaction was often put down to other causes, such as a “stomach bug”, and may even have been forgotten. It was only after the affected individual suffered a similar reaction days, weeks or months later, after another meal containing mycoprotein, that the foodstuff was identified as a common factor. In

the spirit of experiment, some people even self-administered a small amount of mycoprotein as a challenge in order to provoke symptoms.

The number of individuals affected by these reactions is very small and has remained stable at around 200 reported cases per annum (Dr G. Rodgers, personal communication). However, in the present political climate, the food industry as a whole is under close scrutiny. Thus, any link between a foodstuff, particularly a novel foodstuff, and health matters, excites considerable interest in the public domain.

Prompted by concerns raised by the affected individuals, along with requests for any information regarding possible underlying mechanisms, Marlow Foods Ltd. approached Professor Anne Ferguson, late Professor of Gastroenterology at the Western General Hospital, Edinburgh, to commission a research project to discover the cause of these adverse reactions. This approach also coincided with the company's plans to market the mycoprotein based foodstuff in the United States and the requirement by the American Food and Drug Administration for evidence of its safety in humans.

If one reads through a selection of these letters the most striking feature to note is the remarkable similarity of the reactions described, not just between different individuals but also within the same individual on different occasions. These similarities imply that there is some common organic process underlying these reactions. The nature of these reactions varied but could be placed in one of three broad categories, namely:

- *Anaphylactic-type reaction.* This was potentially the most serious type of reaction, but affected only 3% of respondents. Symptoms ranged from peri-oral tingling to swelling of the soft tissues around the eyes, mouth and airway. In some this was associated with breathing difficulties.
- *Generalised skin rash.* Seen in 3% of cases. Rashes described include a generalised erythematous, papular eruption and the classic “wheals” of urticaria.
- *Abdominal pain, bloating and vomiting occurring between two and six hours after ingestion of the product.* This was by far the commonest reaction described, affecting 94% of respondents. Symptoms were relieved very quickly after vomiting and the individuals were not left with any significant residual symptoms thereafter. A small number had pain and bloating without vomiting.

As it was felt that the less common, anaphylactic-type and skin reactions were almost certainly classical IgE-mediated food allergy reactions, attention was focussed on the largest group, those who had suffered the idiosyncratic but remarkably consistent vomiting reaction. Abdominal pain, bloating, vomiting and diarrhoea are all rather non-specific symptoms but are recognised to occur commonly in response to food allergy or food intolerance (10). This reaction may have been caused by sensitivity to mycoprotein or to the egg albumin used as a binding agent. As egg allergy is a commonly found food allergy (11) it was felt initially that this alone might explain the reaction.

A previous study using Radioallergosorbent tests (RAST) and skin-prick testing (12) had investigated specific IgE responses to mycoprotein in the sera of 10 affected subjects and

compared these with 33 production workers employed in the manufacture of the product. Their results were largely negative; no affected subject had a positive RAST to mycoprotein, but 2 of 10 had positive skin-prick tests. 2 of the production workers had positive RAST results (>2% binding) but neither had experienced any adverse reactions to the product.

The clinical history given by affected subjects was suggestive of a direct irritant or toxic effect of the mycoprotein on the gastric mucosa, or of delay in the normal emptying of the stomach. *Fusaria* species are known to produce a variety of mycotoxins including the type A tricothecenes diacetoxyscirpenol (13,14) and scirpentriol, the type B tricothecene, deoxynivalenol, and zearalenone (15). When ingested they can cause vomiting, diarrhoea, cardiac and central nervous system abnormalities. These mycotoxins are particularly toxic to mammals and are known to be capable of inhibiting protein synthesis (16). The nature of the toxins is well recognised and during the manufacturing process great care is taken to test for their presence. Any batch of *F. graminearum* showing signs of contamination is immediately discarded (G. Rodger, Marlow Foods, personal communication).

1.3.0 Aims

In this thesis, I propose to present and discuss the investigation of a specific group of individuals with no prior history of gastrointestinal disease, who experienced an idiosyncratic, adverse reaction to a novel, mycoprotein-based foodstuff. The investigation aimed to identify the underlying aetiology of these adverse reactions. The

original hypothesis was that adverse reactions may be due to an immunologically-based, humorally mediated mechanism, directed against a component of the foodstuff. This component may be the mycoprotein itself, or egg albumin used as a binding agent. The alternative hypotheses were that these reactions may be a consequence of a direct irritant effect on the gastric mucosa or a delay in gastric emptying. All three possibilities will be investigated in this study.

By way of introduction, the potential interactions between dietary components and the intestine will be illustrated by describing the nature of the interface between the intestinal mucosal barrier and the luminal contents. I will concentrate mainly on the effects of dietary proteins on the gastrointestinal tract and will describe how interactions between these molecules and the intestinal barrier may manifest clinically as symptoms or signs. The evidence for and against a role for food sensitivity in the causation of irritable bowel syndrome (IBS) and in inflammatory bowel disease (IBD) will be reviewed along with the therapeutic role played by diet in inflammatory bowel disease.

Chapter 2- Literature Review

2.1.0 Possible Mechanisms Underlying Food Reactions

When food borne infection is excluded, reactions to foodstuffs may be categorised broadly into two categories, immunologically and non-immunologically mediated. Any immune response involves first recognising a potential pathogen, followed by mounting an effective reaction against that pathogen to neutralise it. Immune responses may be innate (or non-adaptive), or adaptive. The former is not antigen-specific and does not change with repeated exposure to a particular pathogen or antigen. The latter usually requires an initial priming phase on first exposure to an antigen (primary response), is highly antigen-specific, and will boost the reaction mounted in response to subsequent exposure to that antigen in the future (secondary response).

Acquired or antigen-specific immune responses may be mediated by two different mechanisms: humoral, that is via the effects of circulating proteins or antibodies produced by B-lymphocytes (B-cells) and plasma cells, or cellular, via direct effects of immunologically active cells such as T-lymphocytes (T-cells). Humoral responses may be detected by measuring antigen-specific antibody levels in a body fluid, while cellular responses are more difficult to measure and require skin tests (*in vivo*) or measurements of cell proliferation in response to stimulation by an antigen (*in vitro*).

The recognition of foreign antigens is the hallmark of the adaptive, antigen-specific immune response. Two types of molecule are involved in this process, immunoglobulins, secreted by B-cells, and T-cell antigen receptors (TCRs). Immunoglobulins are a group

of glycoproteins present in serum and other tissue fluids in all mammals, where they may be floating free in blood or lymph, or attached to the surface of B-cells, acting as specific receptors for antigen. There are five major classes of immunoglobulin antibodies. IgA predominates at mucosal surfaces, particularly in the intestinal and genitourinary tracts, but is found in very low concentrations in plasma. IgG is the predominant antibody class found circulating in plasma and is produced mostly in secondary responses to antigens. IgE is present in excess in atopic individuals and is produced in response to allergic reactions. IgM is mostly produced during the initial, primary reaction to an antigen. IgD accounts for less than 1% of the total plasma immunoglobulin but is found in large quantities on the surfaces of many B-cells.

Immunological reactions to dietary antigens, with the production of an antibody response, are common but the nature and intensity of any clinical reaction varies widely. Exposure to a variety of different foods commonly leads to the production of specific IgG antibodies directed against these foods (17-19). Clinically apparent reactions to dietary proteins are rare and in most instances, no pathological consequences ensue. The most commonly seen, and often the most severe reactions tend to be mediated by immunoglobulin-E (IgE). For example, in susceptible or sensitised individuals, exposure to even tiny quantities of peanut may trigger urticarial skin rashes, oral allergy syndrome or less commonly, but potentially fatal anaphylaxis.

Other immunological reactions to foods, not mediated by IgE are thought to be triggered by exposure of immunologically competent cells including macrophages and T-lymphocytes to particular dietary proteins. These cells produce a variety of pro-

inflammatory cytokines, such as tumour necrosis factor- α (TNF- α), which in turn precipitate and perpetuate inflammation. Lymphocyte stimulation experiments showed that peripheral blood lymphocytes from infants with cow's milk protein allergy produced significantly higher levels of TNF- α than non-allergic controls (20). The delayed reaction to ingested dietary gluten seen in Coeliac Disease and Dermatitis Herpetiformis does not involve IgE but is mediated by other immune mechanisms, mainly T-helper₁ cell mediated, with recruitment of large numbers of lymphocytes into the epithelial compartment of the small intestine. These autoimmune conditions are known to have a strong genetic basis and are most commonly associated with the human leukocyte antigen (HLA) DQA1*0501 and DQB1*0201 alleles. This HLA-DQ2 allelic combination is found in 98% of Northern European Coeliac patients (2,21). Furthermore, specific peptides of gliadin have been identified, which trigger an inflammatory T-cell response (22).

Reactions which have no immunological basis may be regarded as food intolerances rather than true allergies. These can be defined as any abnormal response to an ingested food which is neither psychologically nor immunologically based. Pharmacological reactions to foods can occur, either by direct pharmacological or biochemical actions of a constituent of the food, e.g. caffeine, tyramine in cheeses, or by the non-specific, non-immune mediated release of pharmacologically active substances, such as histamine. Other mechanisms of intolerance include the specific inability to digest elements of the foodstuff adequately, as is the case in disaccharidase deficiencies such as lactase deficiency. Defining dietary components as antigens confers immediately an idea of a

reaction between these components and the intestinal immune system. In order to get a better understanding of the potential reactions between these two entities we must look in more detail at the structure and function of the intestinal mucosa itself.

2.2.0 Interface Between Luminal Contents And The Intestinal Mucosa

The intestinal mucosa constitutes the body's largest surface area, and is very much in the front line in terms of exposure to environmental antigens, bearing the heaviest burden of exposure to such antigens. Its structure reflects its dual role as a barrier to potentially harmful agents while allowing passive and active uptake of essential nutrients and selective "sampling" of luminal contents. The intestinal immune system is composed of finely tuned, extensively interacting populations of cells and their products. These are involved in both the innate and specific immune defence against ingested antigens. There is a constant compromise between the need to absorb adequate nutrients from food and at the same time exclude any harmful pathogens. The massive surface area and absorptive capacity of the intestine means it is constantly exposed to huge numbers of potentially antigenic substances. The mucosal barrier exists to limit some of this interaction and prevent potentially pathological reactions.

Controlled uptake of luminal macromolecules allows delivery of nutrients and growth factors to the peripheral circulation and constant sampling of luminal antigens. However, it is possible that excessive or non-specific uptake of such luminal molecules may trigger immune reactions outwith the local response, recruiting large numbers of immunologically competent cells, such as macrophages and T-lymphocytes which, may

lead to damage to the intestinal epithelium. The “mucosal barrier” is made up of several components, some providing physical and others immunological protection. Non-specific protective mechanisms include the degradative actions of gastric acid and pancreatic digestive enzymes which begin the process of breaking down large, potentially antigenic molecules, to form smaller molecules with less antigenic potential. The continuous peristaltic activity of the gastrointestinal tract means that luminal contents are constantly being propelled along in an aboral direction, ensuring adequate mixing with digestive juices and limiting the time for which they are in contact with any particular point on the luminal surface. In addition, mucus and immunoglobulin A (IgA) are secreted onto the luminal surface.

Mucus Intestinal mucus consists mainly of mucin glycoproteins which form a viscous coating over the epithelial cells, lubricating its surface and providing a thick physical barrier. These glycoproteins consist of a protein core bound by N-acetylgalactosamine links to a variety of complex carbohydrate side chains, which may constitute up to 80% of the total molecular weight. They are produced in the endoplasmic reticulum of mucosal goblet cells, packaged by the Golgi apparatus and secreted from the apical surface of the cell into the intestinal lumen. Here they provide a non-specific line of defence against potential pathogens in the intestinal lumen. Mucus secretion can increase in response to irritants, thus increasing the thickness of the protective layer.

Over the past decade, twelve genes involved in coding for mucin proteins have been described (23,24). They are categorised depending on their function as visco-elastic

polymers or location as membrane anchored molecules in the cellular glycocalyx. Two clusters have been identified on chromosome 11p15.5 and on chromosome 7q22. A thinner mucus layer is seen in ulcerative colitis compared with normal colon or in Crohn's disease (25). In addition, sulphation of mucin tends to be reduced and this correlates with the severity of inflammation (26,27).

Mucus can interfere with adherence of certain antigens and micro-organisms to specific carbohydrate receptors on the apical membrane of the epithelial cells thus inhibiting their pathogenicity. Mucus can similarly inhibit binding of lectins, carbohydrate-binding proteins derived from plants and bacteria, which can activate T-cells via their TCRs, or macrophages via cross linkage of IgE molecules. There is a possibility that competition occurs between mucin carbohydrates, micro-organisms and antigens for specific binding sites and subsequent uptake into epithelial cells.

IgA IgA is present in small amounts in the circulation but is much more abundant in intestinal secretions. There is a huge predominance of IgA-producing cells in the intestine and in intestinal fluids. It is the most heterogenous of all the immunoglobulins, occurring in two distinct subclasses, IgA1 and IgA2 and in different molecular forms; monomeric, polymeric and secretory (28,29), all of which are distributed differently between the circulating immune system and the mucosal immune system. Different molecular forms of IgA may have different functional properties, and these properties are also site-specific. Secretory IgA has several advantages over other IgA isotypes. It possesses between four and eight antigen binding sites, allowing more efficient antigen

binding (30,31). It is more resistant to proteolytic degradation because of the structure of its α chains and through a protective effect of secretory component (SC), the major extracellular fragment of the epithelial polymeric immunoglobulin receptor (pIgR), which remains covalently bonded to polymeric IgA and prevents proteolysis during its transcytosis through epithelial cells. Unlike other immunoglobulin classes, IgA displays potent anti-inflammatory properties. Intact, native human IgA antibodies do not activate complement when complexed with antigen (32). Indeed, IgA appears to have an inhibitory effect on the complement-fixing and complement-activating properties of both IgG and IgM class antibodies (33,34). The protective effect of secretory IgA depends, in part, on its ability to inhibit the absorption of both soluble and particulate antigens within the lumen by complexing with them, neutralising infective antigens, such as viruses, and binding to other microbial antigens to prevent their adherence to epithelial cells. One mechanism by which this is achieved is through the chemical structure of the IgA molecule, whose Fc region is rich in mannose-type glycan side chains. These interact with mannose binding lectin on the fimbrial processes of enterobacteria, causing bacterial aggregation and thus preventing bacterial adherence to similar mannose rich residues on intestinal epithelial cells.

Perhaps the best known clinical example of the protective, anti-inflammatory effect of IgA is that of the passive immunising effect of pre-formed secretory IgA (s-IgA) antibodies, produced through maternal exposure to microbial antigens, ingested by infants in maternal breast-milk, which protects them from subsequent gastrointestinal infections. This natural protective effect of maternal pre-formed IgA has been exploited

further by feeding low birth weight infants a formula fortified with a very high concentration (70%) of IgA to prevent the devastating complication of necrotising enterocolitis (35).

Intestinal Epithelium The entire intestinal tract is lined by a single layer of epithelial cells making them the most numerous cell population within the intestinal mucosa. There are four main types: columnar, polarised epithelial cells, capable of vectorial transport of nutrients and electrolytes; mucosal endocrine cells; goblet cells, capable of producing and secreting mucus and Paneth cells, capable of producing defensins. All gut epithelial cells originate from a population of stable stem-cells located near the base of crypts. They undergo differentiation as they migrate up the crypt-villous axis. As they reach the tip of the villi they will undergo apoptosis and be sloughed off into the intestinal lumen. This whole process takes between three and five days.

2.3.0 Reactions Between Luminal Contents And The Intestinal Immune System

2.3.1 Antigen Uptake By Enterocytes

Enterocytes in mammals of various ages have been shown to take up macromolecules from the gut lumen by endocytosis (36–40). The molecules attach to the apical surfaces of the enterocytes to be taken up whole within an endosome which may then be

processed within the cell. Tiny amounts of unprocessed antigen may pass out into the interstitial space by exocytosis from the basolateral membrane of the enterocyte (41).

Cornell *et al*, using Horse Radish Peroxidase (HRP) injected into ligated loops of rat intestine, were able to demonstrate the presence of particles in the apical membrane of the enterocyte, within membrane-bound cytoplasmic structures, between enterocytes and finally in the lamina propria, indicating transcellular, non-specific transport of macromolecules across the enterocytes (38).

Specific surface receptor-mediated transport across the enterocyte can also be shown in mammalian models. For example, intact IgG in ingested milk has been shown to be taken up by a Fc-receptor mediated process facilitated by low pH in the gut lumen (42). Similar receptor-mediated processes may be important for uptake of trophic substances such as Epidermal Growth Factor (EGF). However, the EGF receptor lies on the basolateral aspect of the enterocyte, implying that EGF would have to get across the epithelium before binding to it (43,44).

2.3.2 M-cells

Membranous epithelial cells or M-cells are specialised for transport of macromolecules across the intestinal mucosal barrier. They are so-called because of the wrinkled appearance of the apical surface of the cell, seen on electron microscopy, due to numerous microfolds (45,46). Uptake of whole macromolecules is necessary for the generation of secretory immune responses such as IgA. This population of cells has evolved to allow controlled transfer of macromolecules across the mucosa. They are

found in greatest numbers overlying lymphoid follicles in the intestine e.g. over Peyer's patches in the terminal ileum. They differ in structure from the surrounding enterocytes. Those components of the enterocyte, which comprise the normal barrier function, are less well developed in M-cells. There are relatively few microvilli on their apical surfaces and correspondingly little of the glycocalyx that typifies enterocytes. There is considerable heterogeneity of M-cells with respect to their expression of polysaccharides of membrane bound glycoproteins and glycolipids. The pattern of glycosylation may differ from that in surrounding enterocytes. Human M-cells express the sialyl Lewis A antigen (47), which can be used as a marker of M-cells. In addition, the usual layer of overlying mucus tends to be thinner and hydrolytic enzymes, such as alkaline phosphatase, which are present in the microvillous brush border of enterocytes are reduced or absent in M-cells. This lack of alkaline phosphatase can be used to identify M-cells by light microscopy, although this technique may be unreliable since other follicular associated epithelial cells may also be deficient in this enzyme (48). Intracellularly, lysosomal enzymatic activity is reduced. On the basal aspect of the M-cell there is a deep invagination of the cellular membrane (often called the M-cell pocket) into which cells of the mucosal immune system can intrude. These cells are separated from the apical membrane by only a very thin rim of intracellular cytoplasm allowing the lymphocytes and macrophages of the intestinal immune system to rest close to the contents of the intestinal lumen.

A number of pathogenic micro-organisms including *Vibrio cholerae*, *Campylobacter jejuni* and *Shigella flexneri* (49) are known to be transported across the mucosa by M-

cells. They may also be of significance in the uptake of other infective agents, such as prions (50). Horse radish peroxidase (HRP) has been used as a marker of macromolecular transport. Despite the adaptation of the M-cell for efficient macromolecular transport, as demonstrated by studies using uptake of HRP (51), the total absorptive surface area of these cells is small when compared to the vast absorptive area of the mucosal surface taken as a whole. We know that in the face of increased concentrations of HRP, enterocytes are also capable of significant uptake, so it is likely that the relative importance of these two pathways will depend on the amount of antigen within the lumen.

2.3.3 Paracellular uptake

This represents a “third way” for passage of potential antigens from the intestinal lumen to the immunologically active cells in the lamina propria. It has long been appreciated that water and electrolytes may pass between cells but only more recently was it discovered that other larger soluble molecules may also cross the intestinal barrier by this route. Indeed, the paracellular route is the dominant path for the passive flow of solute across the intestinal epithelial barrier (52).

The major barrier to paracellular uptake is the tight junction, which is capable of selectively controlling the diffusion of ions and small, water-soluble molecules from the intestinal lumen to the bloodstream. This selective control allows for counter-regulation of concentration or ionic gradients across the epithelial barrier which may arise as a consequence of transcellular transport mechanisms (53). The anatomical structure of the

tight junction has been studied in some detail. In freeze fracture preparations, (54), these structures are seen to be composed of strand-like filaments passing between cells. The precise composition of these filaments is unknown but the number of strands within a tight junction determines the ionic resistance of the epithelial monolayer, and thus, the degree of permeability by the paracellular route.

Using the technique of solvent drag, Pappenheimer showed that the rate of uptake of molecules less than 5500 Daltons was proportional to the rate of fluid absorption (55-57). He calculated that the size of the pore at an open tight junction to be 5 nm (54), similar to the size of small macromolecules. The passage of larger polypeptide molecules may be facilitated by sodium-glucose transport, but significantly larger, potentially immunogenic macromolecules (such as HRP) will not pass tight junctions under physiological conditions. In the healthy intestine, tight junctions are impermeable to HRP.

Tight junctions are dynamic structures and their function can change rapidly in response to the prevailing physiological and pathological circumstances. More recently, Fasano *et al* have done much to elucidate the possible mechanisms by which tight junction permeability is regulated. The zonula occludens toxin (Zot) produced by *Vibrio cholerae* and zonulin, an endogenous intestinal Zot analogue, have been shown to bind to a common intestinal receptor on the luminal surface, inducing protein kinase C dependent polymerisation of actin microfilaments within the tight junction complex (58-60). The net effect of this binding is a relative opening or “loosening” of the tight junction, increasing the permeability of the epithelial barrier. The surface receptor is only

expressed in the jejunum and distal ileum, not in the colon (61). This may be a potentially important mechanism in those diseases, such as Coeliac disease, which predominantly affect the small intestine, leading to increased intestinal permeability and thus greater exposure of luminal antigens to antigen-presenting and other immunologically active cells within the lamina propria.

2.4.0 Responses To Luminal Antigen Uptake

In the preceding sections I have described some of the means by which the intestinal immune system takes up or samples antigens within the lumen, and exposes them to the immuno-competent cells within the lamina propria. Once antigen has been taken up it must be processed and presented in an acceptable form to immunologically competent cells such as T-lymphocytes in the intra-epithelial or lamina propria compartments. Distinct populations of specialised antigen presenting cells (APCs), usually macrophages, dendritic cells and B-cells accomplish this presentation of exogenous antigen.

Prior to their presentation, antigens must first be processed, and this usually occurs either in the macrophage or in epithelial cells. Antigens are not presented by major histocompatibility complex (MHC) as intact proteins but rather as processed peptide fragments at the cell surface. The majority of epitopes recognised by T-cells are fragments from peptide chains which would be inaccessible in terms of immune recognition in the intact protein. Only a minority of peptide fragments from a protein antigen is able to bind to a particular MHC molecule. Different MHC molecules bind different sets of peptides. The peptide binding site on the MHC molecule has a variety of

clefts, hollows and depressions which depend on the precise nature of the amino acids within the binding “groove”. This will vary from one haplotype to the next. Peptide binding depends on the nature of the peptide side chain branches and their complementarity with the MHC molecule’s binding groove.

The processing of intact antigens to generate peptides that can bind to MHC molecules occurs in intracellular organelles following the internalisation of antigen into the cell. Partial proteolytic degradation of the antigen proceeds within the phagolysosome to degrade it into peptide fragments. These are transported through the cell, coming into contact with MHC class II molecules on their way to the surface of the antigen-presenting cell.

CD4⁺ T-helper lymphocytes will recognise cell-processed antigen in association with MHC molecules and can only be stimulated by antigen which is presented to them by cells expressing MHC class II on the cell surface. Cells expressing MHC class II (APCs) present antigen to CD4⁺ T-cells and this is the route for exogenous antigens taken up from the mucosal surfaces or blood. In contrast, cells expressing MHC class I (which includes all nucleated cells) will present antigen to CD8⁺ T-cells, and this is especially important for responses to intracellular pathogens such as viruses.

Dendritic cells have been described as the most important single population of cells within the immune system although since they are an extremely heterogeneous cell group, and exist in a huge number of different forms, it may be misleading to label them as a

“single” group. Not only do dendritic cells process and present luminal antigens which have succeeded in crossing the epithelial barrier, they are capable of directly sampling luminal contents. Using epithelial cell monolayers, Rescigno *et al* have demonstrated that this is achieved through the expression of tight junction proteins on the dendritic cell surface. Through their interactions between the dendritic cell and epithelial tight junctions, these proteins are then exploited by allowing dendritic processes to extrude through the epithelial barrier, between the tight junctions and into the lumen, while still maintaining the integrity of the epithelial layer (62).

It has been postulated that in inflammatory bowel disease, (63) the presentation of certain luminal antigens such as bacteria or bacterial cell wall products e.g. lipopolysaccharide (LPS), or even dietary proteins, may trigger increased expression of MHC class II molecules on epithelial cells. This in turn increases activation of macrophages and other antigen presenting cells. This increased presentation of antigen to immunologically competent cells may increase activation and expansion of CD4⁺ T-Helper lymphocytes. This further encourages T-cell activity, enhances proliferation and maturation of B-cells and ultimately can increase the numbers of immunoglobulin secreting plasma cells.

Further insight into the importance of the innate immune response to luminal antigens, particularly that of bacterial cell wall LPS, in the aetiology of Crohn's disease was gained earlier this year from genetic studies. Groups working in Europe and the USA described genetic defects in the Crohn's disease susceptibility locus on Chromosome 16. These genetic variants, code for a variant NOD2 protein which, acting via nuclear transcription

factors such as nuclear factor- κ B (NF- κ B) in monocytes may lead to over activation of the intestinal cellular immune response to intra luminal bacteria (64-66).

2.5.0 Generation Of Antibodies Directed Against Dietary Antigens

Antigen uptake by the mammalian intestine is influenced by age, and by the stage of maturation of the intestine. Humans differ from other mammals in that the intestinal tract is at a relatively advanced stage of maturation at the time of birth. This means that the human neonate is theoretically less able to absorb large macromolecules such as maternal immunoglobulins in colostrum than for example the calf (67). This is of no functional significance as, in humans, passive maternal immunity is passed on through transplacental transfer of immunoglobulin *in utero*. However, increased levels of macromolecules such as Bovine Serum Albumin (BSA) do penetrate the human neonatal intestine and can be measured in serum. This is not the case in older children fed equivalent doses of BSA (68). Other studies (37,69,70) have confirmed this finding. They show that there are higher levels of detectable antibodies directed against food antigens in the first three months of life than in later infancy. This suggests that proteins can pass across the intestinal mucosa more easily in earlier life, but also that there may come a point at which this passage ceases or diminishes significantly, a point known as “closure” of the intestine. Whether this concept is relevant in humans is debatable in view of findings which conflict with the above. For instance, Robertson *et al* concluded that closure was complete in humans at birth. They were unable to demonstrate bovine β -lactoglobulin in the sera of term human neonates fed on bovine milk (71). Many other

authors have demonstrated detectable levels of IgA class antibodies in serum directed against food antigens such as egg albumin and cow's milk proteins in adults (17,18). At least some of the differences encountered may relate to the methods employed to look for antibodies with a variety of techniques being used over the years including radioimmunoassay (RIA), and enzyme-linked immunosorbent assay (ELISA). However, the presence of antibodies directed against dietary components suggests that the process of "closure" is not as simple as previously supposed and is probably never totally complete. This situation allows sampling of antigens in the intestinal lumen to persist long into adulthood. Recent electron micrograph studies have demonstrated that dendritic cells can extrude into the intestinal lumen, coming into contact with the antigenic material contained therein (72). Thus, increases in intestinal permeability may result in not only increased movement of luminal contents across the mucosal barrier to the lamina propria, but also a greater number of lamina propria dendritic cells sampling luminal antigens. The situation is not one of a simple one-way barrier, which is either open or closed, but allows a constant traffic to cross to and fro in a controlled manner.

De Weck *et al* described a high prevalence of IgG antibodies to milk, egg, soybean, peanut and fish mix in a group of 105 healthy male blood donors aged between 20 and 25 (73). A number of other authors have found higher levels of IgG class antibodies to cow's milk in the sera of cow's milk allergic patients but this is not a consistent finding (74-80). It is most likely to be a reflection of the physiological response of the intestinal immune system to exposure to substances in the lumen. However, when compared with normal subjects, increased levels of antibodies to many different dietary substances have been reported in individuals with inflammatory disorders of the intestine including

Coeliac disease, Crohn's disease and Ulcerative colitis. In the majority of the earlier reports, authors have concluded that the presence of these antibodies is due to increased permeability of the mucosal barrier as a consequence of intestinal inflammation(81). However, other mechanisms may be at play, such as a break down of the normal protective mechanisms which prevent excessive activation of the mucosal and systemic immune systems in response to ubiquitous dietary components.

Following antigenic stimulation, lymphocytes in Peyer's patches migrate to mesenteric lymph nodes via lymphatic channels. From here they travel in the lymphatic system to the systemic circulation. Suitably activated or sensitised cells will then "home" to sites which will facilitate their interaction with absorbed antigen such as the intra-epithelial lymphocyte compartment and the lamina propria.

It must be noted that the mucosal immune system is largely independent of the systemic immune system and that responses to antigenic stimuli are also different. For example, there is a relative lack of IgG producing plasma cells within the intestinal mucosa and so mucosal stimulation will tend to produce the local secretion of IgA class antibodies. Systemic responses are characterised by secretion of IgG, the most abundant immunoglobulin in the blood. As a result of the enormous quantity and diversity of antigen presented to the mucosal immune system, in the form of ingested or inhaled material, robust systems must exist to avoid excessive or inappropriate immune responses to innocuous substances. These mechanisms will be discussed in more detail in section 2.6.0.

The investigation of intestinal mucosal humoral immunity presents particular logistical problems not least of which is the relative inaccessibility of the vast majority of the intestinal mucosal surface. Investigators have attempted to study intestinal mucosal immunity in a variety of ways by measuring immunoglobulins and specific antibodies in intestinal secretions. This generally involves intubation of the gastrointestinal tract to allow perfusion of isolated segments of small intestine, aspiration of small intestinal juices or the taking of biopsies of intestinal mucosa. Such methods are, by their very nature, invasive and unpleasant for the subject under examination. Largely for these reasons, investigators historically tried to extrapolate data obtained from serum, which indicated a systemic immune response, as representative of events occurring in the gut. This may be a valid method in certain circumstances, such as described by Forrest in the study of IgA secreting B cells or plasma cells following enteric infections or immunisation (82). However, it is potentially misleading to make such extrapolations, and wherever possible, efforts should be made to directly study events at a mucosal level.

Without recourse to intestinal intubation, the only methods available for doing this adequately involve measurement of substances excreted in intestinal secretions or faeces. This is not a problem if one is measuring substances which are robust, stable and do not degrade readily. In view of the plethora of digestive enzymes and fermenting bacteria within the digestive tract which may denature or inactivate proteins in the form of antibodies, enzymes or immunoglobulins, such techniques may present major technical

challenges. Similarly, intestinal transit times, if very long, may result in the denaturing of a number of unstable proteins.

However, the technique of whole gut lavage, using a polyethylene glycol (PEG) solution identical to that used in pre-operative bowel preparation, first described by Gaspari *et al* (83), offers a relatively non-invasive means of assessing intestinal levels of a variety of proteins. O'Mahony *et al*, working in Edinburgh, published extensively on the use of this technique. They were able to quantify the levels of total immunoglobulins (IgA, IgG and IgM) in addition to detecting specific antibodies to gliadin, other common food proteins and a number of bacteria in the intestinal secretions of immunologically normal healthy control subjects as well as in those with Crohn's and coeliac disease, who had disordered intestinal immunity (84-88). Furthermore, the same group went on to show that the elevated levels of whole gut lavage fluid proteins such as IgG and α 1-antitrypsin seen in patients with active inflammatory bowel disease, also correlated well with other clinical assessments of disease activity (89).

A number of very interesting observations arise from the use of this novel technique, reflecting the differences between systemic immune responses, as measured in serum, and mucosal immune responses, measured in whole gut lavage fluid (WGLF). In an attempt to characterise the systemic and immune responses to common food proteins, paired samples of serum and WGLF were obtained from 12 healthy volunteers and a rather heterogeneous group of 54 patients with Crohn's disease, ulcerative colitis and coeliac disease. These were assayed for serum IgG antibodies and WGLF IgA antibodies

to gliadin, ovalbumin and β -lactoglobulin (88). There was no reciprocal relationship between serum IgG and intestinal IgA levels in either group, indicating that the presence of systemic antibodies did not reflect a lack or absence of mucosal IgA response. There were no healthy controls who had intestinal IgA antibodies to any of the foods tested with no detectable serum IgG response. Very few of the disease controls had positive intestinal IgA antibodies in the absence of positive serum IgG antibodies, but the majority of those who did were coeliac patients. In the disease control group, 42% of coeliac patients had this pattern compared to 6% of Crohn's and 3% of ulcerative colitis patients. Enhanced intestinal antibody production to gliadin and other common food antigens in coeliac patients had previously been demonstrated and may represent a breakdown of the usual tolerogenic protective mechanisms active within the mucosal immune system. Levels of serum IgA and IgG antibodies to gliadin were seen to fall in response to treatment with a gluten free diet, while IgA antibodies in jejunal aspirates and WGLF remained high. This highlighted a dissociation between the systemic and mucosal humoral immune responses to gliadin in patients with coeliac disease. Although there was a correlation between levels of serum and jejunal aspirate antibody levels in the untreated coeliacs ($r=0.68$, $p<0.001$), this was lost after withdrawal of gluten (84). In a study of the systemic and mucosal immune responses to *Klebsiella pneumoniae* in two groups of patients with Crohn's disease and ankylosing spondylitis, there was no correlation between serum and WGLF levels of IgG antibodies to *K. pneumoniae* in the Crohn's patients. This further illustrates the potential pitfalls of extrapolating information from systemic antibody levels as a reflection of intestinal mucosal responses (87).

In immunologically healthy individuals, most interactions between antigen absorbed from the intestinal lumen and immuno-competent T cells will usually result in a state of immune hypo-reactivity or tolerance, thus reducing the potentially harmful effects of the absorbed antigen. Only very rarely will a state of hypersensitivity result.

2.6.0 Oral tolerance

The ability of the mammalian immune system to generate appropriate responses to the almost infinite variety of potential allergens it may encounter depends on the expression of vast numbers of immuno-competent cells with different antigen-specific lymphocyte receptors. In so doing, the risk of expressing receptors which will attack “self” proteins, and developing a state of auto-immunity, or of expressing receptors which will attack otherwise harmless, often useful exogenous “antigens”, such as food proteins, is relatively high. However, this is the price we must pay for an immune response which is effective against any potentially antigenic substance. In order to minimise this risk, a number of strategies exist to induce or to facilitate a state of immunological tolerance.

This is achieved by two main strategies, namely, central and peripheral tolerance. Central tolerance is the better understood mechanism and involves the physical destruction or deletion of self-reactive T and B lymphocytes (90). However, this central strategy alone is insufficient to account for antigens which may not be represented in the primary lymphoid organs or which are only found in certain peripheral sites. Therefore

central tolerance has to be complemented by peripheral mechanisms of tolerance, of which oral tolerance is one such mechanism.

For many years it has been known that exposure of the immune system, via the mucosal (oral or nasal) route to soluble dietary antigens leads to a state of immune hyporesponsiveness or tolerance to that specific antigen (91), even when the immune system re-encounters that antigen systemically. It is a protective mechanism to limit potentially harmful immunologically mediated damage triggered by otherwise innocuous substances encountered in the intestinal lumen.

Whether an antigen will induce tolerance in the periphery is probably dependent on the balance of co-stimulatory signals to the T cells from other antigen presenting cells (APCs). The form taken by any particular antigen is also important in determining whether an immunogenic or a tolerogenic response is elicited. For example, antigen presented in soluble, monomeric form is likely to induce tolerance and immune hyporesponsiveness if subsequently encountered. In contrast, antigen presented in particulate form, especially if administered in the presence of an adjuvant is more likely to induce production of pro-inflammatory cytokines including TNF and IL-1, along with upregulation of co-stimulatory molecule expression (such as B7.1 and B7.2), ultimately resulting in an immune response on subsequent challenge (92).

The non-specific effects of digestion of potential antigens, coupled with the combination of intestinal mucus and the intestinal epithelial barrier may be one way of denying potential luminal antigens access to the lamina propria and APCs. T cell clones may be

unresponsive to antigen if they have never encountered it and are immunologically “ignorant” or naive. However, the available evidence would suggest that such a possibility is unlikely, as the intestinal “barrier” is not an impermeable structure. Indeed, oral tolerance can only be induced if the appropriate T cell clones do encounter luminal antigen. The non-specific barrier effect of the gut wall may instead act as a filter, allowing highly soluble antigens, perhaps in monomeric form, access to the relevant APCs and T cell clones and inducing T cell anergy.

Up-regulation of production of secretory IgA antibody production occurs in order to deal with ingested antigen within the intestinal lumen. In addition, there has to be suppression of the systemic immune response to that antigen. This suppressive effect may be achieved in several ways. Stimulating T cell clones via their TCRs, may induce a state of immune unresponsiveness or clonal anergy. Whitacre *et al* demonstrated that, in an encephalitis model, this state can be reversed *in vitro* by the administration of exogenous IL-2 (93), indicating that hyporesponsive T cells do still retain their ability to respond to antigen. Alternatively, T-cell clones may be physically eliminated or deleted, either through apoptosis (programmed cell death) or through release of toxic cytokines and deprivation of cell growth factors. All the available evidence for T cell deletion is derived from animal studies with conflicting results and there is still controversy as to whether apoptosis mediated cell deletion is truly a relevant phenomenon in this context. A further possible mechanism may be via the activation and up-regulation of antigen-specific regulatory CD8⁺ cells. These may produce regulatory cytokines such as transforming growth factor (TGF)- β , which has non-specific suppressive effects on surrounding cells,

regardless of their antigen-specificity (94). However, animal studies utilising genetically engineered, CD8 knockout mice and mice given anti-CD8 antibodies (95) showed that oral tolerance could be induced without difficulty. Therefore it is inferred that the presence of CD8⁺ cells is not an absolute requirement for the induction of oral tolerance, and CD8⁺ cells may play only a minor role. In contrast, CD4⁺ cells appear to play a crucial role, as shown in studies where CD4⁺ cells have been depleted in vivo or in genetically engineered CD4 knockout mice (96). Finally, a number of immunoregulatory cytokines, including IL-4, IL-10 and TGF- β have been studied in relation to the induction of immunological tolerance. Both IL-4 and IL-10, so-called Th2 cytokines have been found to be upregulated in oral tolerance, although they have failed to live up to their initial promise as mediators of this process through the down regulation of Th1 responses (97,98).

Emancipator and Lamb (99) showed that mice orally immunised to bovine gamma globulin or to gliadin developed elevated titres of IgA antibodies to the fed antigen; however, intravenous administration of the same antigens produced elevated titres of IgG and IgM antibodies. A breakdown of the normal mechanisms of oral tolerance is responsible for the lesions seen in Coeliac disease and other food sensitive enteropathies (100). It is possible that some form of impaired tolerance is also implicated in the pathogenesis of inflammatory bowel disease (101-104). For example, Duchmann *et al* were able to demonstrate in a mouse model that normal oral tolerance to intestinal flora could be completely abrogated by the induction of a chemically-induced experimental colitis. In the same model, administration of either exogenous IL-10 or antibodies to the

proinflammatory cytokine IL-12, was observed to restore tolerance. It is not clear, however, whether this was due to any particular tolerogenic effect of the cytokine therapy or rather to their ameliorative effect on the experimentally induced colitis. In either case, induction of experimental colitis in an animal model resulted in a loss of tolerance to the intestinal flora which could be restored by adequate, effective therapy for that condition.

2.7.0 Food Allergy

True food allergy is an abnormal reaction resulting from heightened immunological responses to antigens (usually glycoproteins) in foods. Those foods most commonly implicated include peanuts, soy, cow's milk, eggs, tree nuts, fish and wheat (105). Symptoms are more common in atopic individuals, and severe systemic reactions, such as anaphylaxis are more frequent in asthmatic individuals (106-108), some of whom may also have allergies to pollens.

There is, unfortunately, a lack of a good, robust animal model of food allergy. In spite of this, most of the experimental evidence regarding the pathophysiology of intestinal hypersensitivity reactions is derived from animal studies, particularly rodent studies (10). There are potential problems associated with using a rodent model for the study of reactions occurring in humans. The human and rodent immune systems are not identical and extrapolating data from rodent studies to explain responses in humans is potentially very misleading. Animal studies can provide a paradigm for events occurring in humans, but do not necessarily replicate or even reflect those events. An illustration of the

potential problems encountered when one extrapolates immune responses in the rodent to the human situation is seen in the case of interleukin-3. Interleukin-3 (IL-3) has been shown to be an important regulatory cytokine in rodent mast cell function, but human mast cells do not express the IL-3 receptor (109). Rodent studies therefore highlighted the importance of IL-3 in mast cell function, but to assume that similar mechanisms apply in the human mast cell is obviously a mistake. Where possible, studies involving human tissue are preferable but may not always be possible. In the absence of human material it may be necessary to resort to the rodent model but care must be taken in the interpretation of the data thus obtained. With respect to this study, which aimed to describe the mechanism of a gastrointestinal reaction to mycoprotein in humans, animal models may potentially be misleading.

The mechanism underlying food allergy is thought classically to involve immunoglobulin-E (IgE) mediated aggregation and activation of mast cells and basophils within the affected tissue e.g. the skin, airways or intestine. Mast cells are found throughout the body but fall into two main populations, mucosal mast cells and connective tissue mast cells (110). A very high density of mucosal mast cells is found within the gastrointestinal tract. Intestinal mast cells make up 2-3% of the population of lamina propria cells. Connective tissue mast cells are found adjacent to blood vessels, nerves and lymphatic channels. They are larger than intestinal mast cells and contain more histamine. In connective tissue mast cells, histamine release may be inhibited by administering disodium cromoglycate, which has no effect on intestinal mast cells. In health, mast cells function as part of the host defence mechanism, playing a critical role

in protecting against intestinal infection, especially parasitic and bacterial infections (111,112). Increased numbers of intestinal mucosal mast cells have been described in untreated Coeliac disease (113) and in Crohn's disease. Endoscopically administered antigen provocation tests in humans, with injection of antigen into the gastric (114,115) or caecal (116) mucosa, have revealed elevated levels of tissue histamine and increased mast cell numbers in biopsies taken from allergic subjects. Such information reinforces the importance of these cells and their mediators in the pathogenesis of intestinal allergic reactions.

Cross-linking of antigen or anti-IgE to IgE bound to specific receptors on these specialised cells, causes them to "degranulate" releasing a cocktail of biologically active substances, including histamine, prostaglandins, leukotrienes and tumour necrosis factor- α (TNF- α). These in turn induce an inflammatory response with a number of consequences within the gut, such as increased epithelial ion transport, increased vascular permeability, impaired integrity of the mucosal barrier, mucosal oedema, increased mucus secretion and increased peristaltic activity (117,118). Clinically, this may manifest as abdominal pain, bloating and diarrhoea. Reactions may not occur after first exposure, when the subject becomes sensitised to an allergen, but may be apparent on subsequent exposures. They then usually occur immediately on contact with the allergen but occasionally may be delayed in time. Since mast cell activation in both the connective tissue and mucosal compartments is dependent on IgE-mediated mechanisms, those individuals with high levels of circulating total and antigen-specific IgE are most

likely to experience the clinical manifestations of atopic disease whether in the skin, airways or nasal and gastrointestinal mucosa.

Mast cells are a major effector cell type in IgE-mediated Type 1 hypersensitivity reactions. Stem cell factor (SCF) has been shown to regulate the development of human mast cells from primitive progenitor cells (119). In addition, it enhances the release of histamine and leukotrienes from human mast cells in the lung (120,121) and gastrointestinal tract (122) and selectively promotes survival of gastrointestinal mast cells. In the presence of SCF, interleukin-4 (IL-4), a T-cell derived cytokine, which acts as a B-cell growth factor, enhances the proliferation and survival of mast cells and increases the IgE dependent release of inflammatory mediators (123). However, in the absence of SCF, IL-4 has little discernible effect on human mast cells. Human mast cells also constitutively produce small amounts of the potent, pro-inflammatory cytokine, TNF- α (124) which may be important in their protective role against micro-organisms. However, cross linkage of IgE stimulates increased production of TNF- α and this may account for some of the heightened inflammatory response seen in allergic individuals exposed to allergens. IgE cross linking also stimulates the secretion of other cytokines including IL-3, IL-5 and IL-13 which serve to attract other cell populations, such as eosinophils, to the site of inflammation (125,126). Non- IgE mediated mechanisms including activation of IgG₁ receptors (127) and nitric oxide (128) can also activate mast cells leading to degranulation and mediator release in human and rodent models.

Interactions between mast cells and nervous tissue may also be of importance. In the rat trachea, Joos *et al* described the neurokinin-1 (NK-1) receptor mediated release of serotonin from mast cells stimulated by substance P and neurokinins (NKs) (129). In rodents, nerve growth factor (NGF) increases the number of mast cells and primes them for histamine release (130). NGF levels are known to be elevated in the sera of atopic human subjects (131) and this may represent another, neurally mediated pathway leading to histamine release and allergic reactions in susceptible individuals.

Attraction of eosinophils to the site of inflammation is important in the generation and perpetuation of the inflammatory response in non-intestinal allergic conditions (132). However, less is known about the role of these cells in the pathophysiology of intestinal allergic reactions to foods. The discovery that levels of eosinophil granule proteins such as eosinophil cationic protein (ECP) and eosinophil protein X (EPX) are elevated in the faeces of individuals with food allergy (133,134) would support the notion that they are also of great importance in these reactions. Local stimulation of the intestinal mucosa by food antigens in patients who are sensitive to cow's milk protein leads to increased faecal levels of ECP (135). In the same study, the authors noted that eosinophil activation occurred rapidly, usually within 20 minutes of allergen exposure. Just how eosinophils are activated is not clear. There may be a direct stimulating effect of the offending allergen leading to cross-linking of IgE or IgA receptors on the cell surface. Alternatively, eosinophil activation may be indirectly regulated by the activation of mast cells, leading to increased release of chemoattractants including prostaglandins and histamine, recruiting eosinophils to the affected area (136). A number of cell types

including fibroblasts, macrophages, monocytes, T-cells and endothelial cells can secrete polypeptide chemotactic cytokines which act as powerful chemoattractants. Macrophage chemotactic protein (MCP) -2 and -3, macrophage inflammatory protein 1- α (MIP-1 α), RANTES and eotaxins 1 and 2 can bind to the chemokine receptor 3 (CCR-3) expressed on eosinophils (137,138) where they may trigger release of further inflammatory mediators or merely function as chemokines. The release of other cytokines, such as IL-3, IL-5 and granulocyte/macrophage colony stimulating factor (GM-CSF) has been shown to be of great importance in allergic inflammation, particularly in late phase allergic inflammation (139). IL-3, IL-5 and GM-CSF enhance the release of IgE-independent inflammatory mediators from human eosinophils (140,141) contributing to increased vascular permeability, mucosal oedema and recruiting more inflammatory effector cells to the site of inflammation. Hogan and co-workers (142) described a new mouse model of antigen-induced gastrointestinal allergy which illustrates the role of eotaxin in such reactions. IL-5- or eotaxin- knock-out mice, deficient in either IL-5 or eotaxin, a cytokine constitutively expressed in the intestine, were studied to assess the relative roles of these cytokines. After oral administration of food antigens, both groups developed increased levels of IgE. However, the eotaxin-deficient mice had no evidence of eosinophil accumulation in the gastrointestinal tract, while the IL-5-deficient group had an attenuated eosinophil accumulation compared with controls. However, as previously discussed, it remains to be seen whether such murine studies reliably parallel the events in humans.

Some food glycoproteins are known to be more allergenic than others but the reasons underlying this are far from clear. It may be due to factors pertaining to the protein itself; its size, solubility, resistance to digestion or the quantity ingested. Differences may reflect prior exposure to that antigen or to an immunologically similar substance. Prior exposure need not be by the oral route but could reflect cross reactivity with an inhaled antigen.

Pollen allergies are well known to occur in association with food allergy to fruits or nuts. Major birch pollen allergen Bet v 1 and birch profilin Bet v 2 share allergenic motifs found in potatoes, carrots, apples, pears and celery (143) and Mad d 1, the major apple allergen shares IgE epitopes with Bet v1, leading to cross sensitisation mediated by specific T helper lymphocytes (144). Other compounds, such as peptides which stimulate T-cells or antigen presenting cells, may be present in the same food which may augment or attenuate the immune response.

Food allergy or hypersensitivity is common in paediatric populations but is an uncommon diagnosis in adults. The prevalence of food allergy depends on the criteria used in its definition, but using blinded oral food challenges, Bock *et al* found that 6% of an unselected population of children had proven evidence of food allergy. Following these children to age three, it was apparent that the in the majority outgrew their allergy (145). Host also found that 85% of infants sensitised to cow's milk protein, lost their sensitivity by the age of three (146). European and Scandinavian studies quote a prevalence of cow's milk protein allergy of between 1.9% and 2.8% (147-149). Eggesbo *et al* describe



a point-prevalence for egg allergy in 2 year old children in Norway of 1.6% (95% CI 1.3-2.0%) (11). This study raises another interesting point; the perceived prevalence of food allergy by lay-people within the wider population is much higher than the actual prevalence, especially when rigorous diagnostic criteria are applied. In the Norwegian study, questionnaires were filled in by parents who were convinced that their child had suffered a reaction to egg. Two thirds of these reactions were indeed verified by either skin prick testing or oral food challenges, but this still leaves a third which were not verified as true allergic reactions. The positive predictive value of a parentally perceived reaction was 50%, if that reaction had been reported on a single occasion. This rose to 100% if the reaction had been reported on three occasions. Although precise data are lacking, it is estimated that <1% of the adult population in the United States is affected by allergic reactions to foods (150).

The diagnosis of true food allergy is challenging as many of the symptoms produced are non-specific and may overlap greatly with symptoms of the irritable bowel syndrome. Diagnosis is largely clinical and depends upon a good history of appropriate symptoms consistently elicited within a defined timescale after ingesting a specific food or food ingredient. It is important to elicit a complete account of which symptoms and signs occurred, which foods were implicated, the precise relationship in time between ingestion and onset of symptoms, the reproducibility of the reaction and some indication of the minimum quantity of the offending food which will precipitate symptoms.

The American Gastroenterological Association has recently published guidelines on the evaluation of food allergy in gastrointestinal disorders in an attempt to provide a rational approach to this area and to standardise practice (151,152). These guidelines reinforce the notion that the diagnosis of food allergy is largely a clinical one. It is based upon a history of an allergic or “allergic-type” reaction which is temporally associated with ingestion of the offending food and backed up by certain *in vivo* or *in vitro* tests. In selected cases, the diagnosis may be confirmed by the resolution of symptoms on an elimination diet and recurrence of symptoms after oral challenge.

There are a number of pointers within the history which are useful in determining whether a reaction is likely to be due to food allergy. The diagnosis is more likely in infants or children than in adults. One of the most reliable clinical correlates of immediate reactions to foods is the presence of a personal or a family history of atopic disease. However, by its very nature, the presence of such a history may be based largely on the patients’ ability to understand and to recall a that a diagnosis of atopy had been made. Further corroborative evidence may, of course, be available in the form of case records (but equally, may not be). A history of atopy, with extraintestinal manifestations (dermatitis, urticaria or asthma), or symptoms of an acute illness, (vomiting or diarrhoea) which occur shortly after ingestion of the suspected food, increases the likelihood of a diagnosis of food allergy. The importance of a family history of atopy, as well as personal history, indicates that IgE production in relation to food exposure may be subject to inherited patterns of IgE synthesis and regulation (153). Evidence exists to point to the inheritance of an isotype specific defect which greatly diminishes the ability

to down-regulate an IgE-mediated response (153). However, not all patients who experience IgE mediated food allergy give a family history of such reactions. Therefore the development of IgE in relation to food exposure is likely to be multifactorial, and not a wholly genetically determined phenomenon.

In spite of its pivotal aetiological role in allergic reactions to foods, particularly with reference to mast cell and eosinophil activation, the measurement of total (ie non-specific) IgE in the serum is only of limited use in the diagnosis of food allergy. It cannot and should not be taken alone as evidence of the presence of a food allergy, or indeed, atopy. Host *et al* did demonstrate that measuring total IgE in the serum of “high-risk” neonates was useful in predicting their subsequent risk of atopy (146) but other studies have reached conflicting conclusions. Hide *et al*, measuring total IgE levels in umbilical cord blood, was unable to demonstrate any useful predictive value of such measurements for atopic risk in neonates, even in the presence of some very high values of total IgE (154).

Those individuals who have specific IgE-class antibodies directed against the suspected food in their serum are also more likely to be suffering from truly allergic reactions to foods. These cases are, however, in the minority (6,155-159).

A search for evidence of elevated IgE, or IgE-dependent pathways is the mainstay of laboratory investigation into allergies at present. This can be achieved simply with minimum discomfort to the patient by means of either RAST *in vitro* testing of serum, or *in vivo* skin prick testing. RAST was developed in the 1970s to look for specific IgE

antibodies in serum to a range of potential allergens. The test has the benefit of allowing the testing of subjects who are taking antihistamine medication, removes the need for provocation testing and its attendant risk of anaphylaxis. However, a number of reviews have highlighted and criticised the poor specificity of RAST testing, and its poor positive predictive value (160,161). RAST testing cannot differentiate whether a reaction is due to the allergen being tested, or to some cross reactivity between that and another, unidentified allergen. In spite of these problems, testing for specific IgE antibodies to foods still has an important role. Sampson *et al* recently prospectively studied sera from 100 consecutive children and adolescents referred for evaluation of food allergy. Sera were analysed for specific IgE antibodies to six common foods using a proprietary quantitative immunoassay, the Pharmacia CAP System FEIA®. Results were compared with those of a carefully taken food history or oral food challenge. They concluded that in the case of symptomatic allergy to egg, fish, peanut and milk, in the paediatric population studied, quantification of specific IgE was a useful test for the diagnosis of food allergy (162). In particular, using previously determined cut-off levels for specific IgE antibodies in serum, RAST testing allowed the clinician to predict correctly the likelihood of a child suffering an allergic reaction to a specific antigen on subsequent double blind placebo controlled oral challenge with that food in 95% of cases. Of the 100 children studied, gastrointestinal reactions occurred in 14 of 21 positive egg challenges, 10 of 21 positive milk challenges, 13 of 21 positive soy challenges and 9 of 17 wheat challenges. Sampson suggests that the utility of such testing lies in the ability to predict who will react to a food challenge and may in future eliminate the need for time-consuming, unpleasant, double-blind, placebo-controlled food challenges in a significant

number of children. From the available data, in children at least, a positive RAST may be seen in two thirds of cases of food allergy manifesting with gastrointestinal symptoms when verified by a food challenge.

Skin prick testing is also useful in evaluating individuals with suspected hypersensitivity reactions to foods. This is in effect the *in vivo* equivalent of the RAST test and detects the presence of systemic, not mucosal antigen-specific IgE. As with RAST testing, patients with gastrointestinal manifestations of food allergy may have negative skin tests, further emphasising the potential role of local mucosal production of IgE in such reactions. Liquid preparations of potential allergens, with appropriate controls are placed on the skin and a tiny puncture made through these preparations. A positive test is indicated by a “wheal and flare” reaction, more than three millimetres in diameter at the puncture site. A negative skin test carries a high negative predictive value (>95%), but, in common with RAST, a poor positive predictive value (<50%) (163,164). Positive results may be used to identify those who require oral food challenges. However, unlike RAST, the skin prick test involves further exposure to the putative allergen and so carries a potential risk of anaphylaxis to the subject. The combination of this risk and the poor positive predictive value of the test means that its use in the diagnosis of food hypersensitivity reactions is limited (160).

IgE-mediated reactions, linked closely to mast cell activation, originally evolved as the main protective mechanism against parasitic infestations (165) but other primarily protective roles may be important. For example, the dramatic mechanical body responses

elicited by IgE/mast cell interaction and activation, such as sneezing, coughing, itching or diarrhoea, represent the body's primary defence mechanism against large particles (as opposed to bacteria or viruses). In spite of the close functional relationship between IgE and mast cells of both the mucosal and connective tissue types, there is little or no information available from human or animal studies on correlations between circulating levels of IgE and measures of mast cell activation. However, Goldman and Proujansky noted positive RAST tests or elevated circulating IgE levels in just over a third of 53 biopsy proven paediatric cases of allergic proctitis or gastroenteritis, with higher levels most often seen in the older children (>2 years) (166). This study is one of the more robust in the literature as it uses histologically proven diagnoses as its benchmark and thus removes much of the subjectivity which surrounds the field of food related gastrointestinal reactions. Whether the elevated levels of specific or total IgE are related to activation of eosinophils or mast cells is not discussed. This is most probably because any such discussion would be merely speculative, since, at present, no test exists to adequately examine or dissect the closely linked, interdependent nature of IgE-mediated mast cell and eosinophil activation within the gut.

Tests for specific IgE antibodies to foods are more likely to be positive when there is a history of symptoms which occur rapidly following antigen ingestion, or in patients who have other atopic diseases. There are few studies which attempt to correlate the results of RAST or specific serum IgE testing with IgE levels in intestinal or other mucosal secretions. One study of subjects with house-dust mite allergic rhinitis who had negative RAST and skin prick testing, found high levels of specific IgE in nasal secretions (167).

Nasal secretion antibody titres did not correlate with serum titres, another example of dissociation between systemic and mucosal immune responses. It has been suggested that a similar local IgE response is important in the gut. IgE levels have been measured in faeces and in the jejunal fluid of children with food allergy and found to correlate poorly with levels of circulating IgE (168,169). These findings support the notion that serum IgE measurements may be a sub-optimal means of assessing patients with a history suggestive of food allergy, and imply that IgE produced locally in the gut ie at the mucosal level, is of more significance. However, the validity and reproducibility of the faecal IgE assay may be questionable, especially in view of the findings of the Edinburgh group. Serial sampling of WGLF revealed negligible quantities of IgA in early, semi-liquid stool samples, but higher, reproducible levels when clear WGLF samples (and hence, a steady state intestinal perfusate) were obtained (85). Such methods are unpleasant for patients and have failed to gain widespread acceptance. It is of interest that although these faecal extract studies were performed and published almost 20 years ago, the recent American Gastroenterological Association technical review on the evaluation of food allergy in gastrointestinal disorders makes no mention of them, far less any recommendation to their use in clinical practice (151,152). While this is not a comment on their scientific value, and is not intended to dismiss the potential importance of locally produced intestinal mucosal IgE, it does reflect their perceived clinical relevance. The utility of measuring IgG antibodies to common foods is more debatable and is unlikely to represent a pathological response. The production of IgG class antibodies against most food antigens is almost certainly a physiological response, which is seldom accompanied by any clinically significant reaction in terms of symptoms (17-19,91). However, high

levels of food-specific IgG antibodies may be indicative of a pathological reaction (170,171) but this is controversial.

There are enthusiasts for more invasive provocation procedures, where samples of the offending food are injected or placed directly onto the luminal surface of the stomach or colon (114-116). These do have the benefit of allowing assessment of any potential allergic reaction at the mucosal level, as opposed to distant site testing with specific IgE in serum or skin prick testing. They are invasive, however, may require general anaesthesia for children and do carry a potential risk of severe anaphylactic reaction to the test substance. Their use is confined to those centres with a special interest in these techniques.

The gold standard for the diagnosis of food allergy remains the double blind placebo controlled food challenge using the suspected food. This was first introduced in the 1970s (172) but many physicians, parents and patients find it impractical and time-consuming.

2.8.0 Food Intolerance

These reactions may be defined as any abnormal response to an ingested food. Reactions may occur as a result of a direct toxic effect or from a pharmacological property of any component of a food. In such cases one might expect that the degree of response will be dependent on the dose ingested but this is not always the case. Reactions may also occur as a result of some abnormal host metabolic response to the ingested food. Like food

allergy, it is a widely held lay-belief that the prevalence of food intolerance is relatively high - we have all experienced some foodstuff which does not agree with us - and the terms are often used interchangeably. A study of American housewives (173) found that one in three believed that at least one member of their household suffered from a food allergy. Two British studies reported similar levels of perceived food reactions (174,175). The earlier study, by Burr (175), based upon a postal questionnaire answered by 475 subjects, described that 14% of males and 18% of females reported adverse gastrointestinal reactions to foods. Young *et al* conducted a more rigorous study of 7500 households in South London and a further 7500 drawn from throughout the United Kingdom. Possible cases were initially identified by means of a postal questionnaire, followed up by interviews and finally evaluated thoroughly by double blind placebo controlled food challenges with cow's milk, hen's egg, wheat, soya, citrus fruit, fish and shellfish. The number of respondents in each group was 10552 and 8328 respectively, giving an overall reply rate of 52.7%. Of these respondents, 20.4% reported food intolerance but, when the results of double blind placebo controlled food challenge with a panel of eight foods are taken into account, the true figure is between 1.4% and 1.8%, depending on how strictly the authors defined a reaction during the challenge period (174). The authors conclude that this discrepancy between true and perceived food intolerances may have significant financial and nutritional consequences as individuals expend time and money in the search for foods which will not cause adverse reactions, or lose weight as a consequence of strict, but unnecessary elimination diets. The prevalence of intolerance to food additives is even lower than that of foods (176), which again runs contrary to that perceived by the media and the wider public.

There is a similar perception among the general public that dietary factors play an important role in other gastrointestinal diseases, particularly IBS, but also IBD. But what evidence actually exists to support notion that food intolerances may be implicated in the aetiology these disorders?

2.9.0 Role Of Dietary Factors In The Aetiology Of Irritable Bowel Syndrome

IBS is a chronic, non-fatal functional disorder of the intestine. The syndrome is defined clinically by the Rome II criteria (177) in individuals who complain of abdominal discomfort or pain for at least 12 weeks (which need not be consecutive) over the preceding 12 months. The discomfort must also satisfy at least two of the following three criteria: (a) relief by defecation; (b) association with an alteration in stool frequency; or (c) alteration in stool form.

Its underlying aetiology remains obscure and is most likely to be multifactorial, involving both physiological and psychological factors. It may be thought of as a disorder of sensory or motor function (or both) within the enteric nervous system, although no consistent abnormalities have as yet been identified. Food sensitivity is commonly perceived by patients as a key factor in their symptom complex and has been examined by a number of authors over the years (178,179). The main evidence that food hypersensitivity may have a role in the aetiology of IBS comes from studies where dietary manipulation has been shown to improve symptoms. Alun Jones *et al* (178) conducted three studies on a total of 25 consecutive patients with a clinical diagnosis of

irritable bowel syndrome in the absence of any other pathology. In the first study, an elimination diet was prescribed in which, all patients were asked to limit their diet to a single meat, a single fruit and distilled or bottled water for one week. Of the 21 patients who followed the diet, 14 experienced an improvement in their symptoms. They were then asked to reintroduce a single food each day to see whether this might provoke symptoms. If a food was thought to cause an adverse effect, this challenge was repeated on three occasions. Foods described as provoking symptoms included wheat, corn, dairy products, coffee, tea and citrus fruits. No patient with a wheat intolerance had Coeliac disease as all underwent jejunal biopsy to exclude this condition. Six patients were then admitted, and for four days followed a diet free of foods which provoked their symptoms. Breakfast on each day was served as a liquidised preparation of a test or control food, in a double blind fashion, via a nasogastric tube. The authors describe significant increases in levels of prostaglandin E2 (PGE2) in rectal dialysates following administration of test foods, not seen on days when control foods were given. There is no mention of whether symptoms were provoked on test days in these patients. In a third experiment, rectal PGE2 levels were only increased in patients who developed diarrhoea. This complicated, time consuming study must have been difficult to perform and presumably recruited a number of highly motivated subjects. It does illustrate two interesting points: (a) that an elimination diet can be effective in reducing the symptoms of irritable bowel in a significant number of patients; and (b) that measurable physiological changes do take place in individuals with IBS when re-challenged, in a blinded manner, with certain foods. Many patients will have tried their own dietary manipulation prior to consulting with medical professionals (180). How much is true allergy and how much is placebo

effect is difficult to tease out. The largest trial to date of dietary manipulation was conducted in Oxford (181). This trial studied a total of 200 patients, with a marked predominance of female subjects (144 female). When subjects were placed on an elimination diet, 96 subjects (48%) reported an improvement in their symptoms. Improvements were maintained for a mean period of 14.7 months. Of the responders, 73 were able to identify one or more foods which would provoke symptoms. However, of the non-responders, 95/98 still complained of symptoms at the final follow up visit, over a year later. We may infer from this that in many, but by no means all, patients suffering from IBS, food intolerance may have some part to play. Identification and avoidance of that food is likely to give sustained relief from symptoms.

A multi-centre European study compared the effect of an elimination diet with that of treatment with oral disodium cromoglycate, a mast cell “stabiliser,” which inhibits degranulation of mast cells and consequent release of inflammatory mediators. In a study of 428 patients, they found that 67% of those treated with cromoglycate experienced symptomatic improvement, comparable to 60% of those on an elimination diet. Of note is the fact that the presence of a positive skin prick test to food allergens predicted a greater response to both modes of treatment (75% vs 54% on elimination diet, and 81% vs 58% on cromoglycate). This might be expected if predominantly IgE mediated mechanisms are at work (182). The results of this study are unusual when one remembers that cromoglycate stabilises connective tissue mast cells, and should have no effect on mucosal mast cells, the cell population which might be expected to be most relevant in intestinal reactions. It is possible that cromoglycate has other actions,

independent of its effects on mast cells. However, it should be pointed out that although this study involved a large number of patients, there was no placebo group against which to measure the effects of both interventions. It is impossible to quantify how much of the observed benefits arose as a result of a placebo effect.

Positive skin prick tests, elevated total serum IgE and food-specific IgE (RAST) have been cited as evidence that IBS may be caused by IgE mediated mechanisms, particularly in atopic patients (183-185). One problem with this hypothesis is that both conditions are very prevalent, particularly in the developed world. Therefore, such positive results may simply reflect the atopic tendency, as opposed to being central to the pathogenesis of intestinal symptoms, in individual patients. In most cases, since the intestine has only a limited repertoire of responses to any form of insult, there is tremendous overlap between the symptoms of IBS and that of food allergy. This can make differentiation between the two conditions difficult. Neri *et al*, working in Italy, described a group of 288 patients attending their clinic complaining of intestinal symptoms. Of these, 99 had IBS and 22 had food allergy, as diagnosed by elimination diet, skin prick tests and RAST. In this study population, at least, symptoms of lower abdominal pain, abdominal pain relieved by defaecation, frequent episodes of abdominal pain and abdominal bloating were all significantly more likely to be found in those with food allergy compared to those with IBS (186).

In summary, food sensitivity may play an important role in the pathogenesis of irritable bowel syndrome in many cases. This is supported by information from elimination diet

studies and treatment with mast cell stabilisers such as cromoglycate. The precise mechanisms by which this occurs remain uncertain, but IgE mediated mast cell activation is likely to play a significant role (10) as it does in atopic conditions like asthma and eczema.

2.10.0 Role Of Dietary Factors In The Aetiology Of Inflammatory Bowel Disease

One of the most commonly asked questions when the diagnosis of inflammatory bowel disease (IBD) is first explained to patients in our out-patients department is, “will I have to stick to a special diet?” This question more than anything else, serves to highlight the link, which exists in the minds of patients and the lay public, between diet and disorders of the gastrointestinal tract. Many patients with IBD remark that the clinical course of their disease is affected by the food they consume. Such a link may seem intuitive but there is surprisingly little evidence to support the idea. There is no clear evidence that dietary factors have any influence in the causation of inflammatory bowel disease, but some interesting epidemiological points have been noted. Perhaps the best known of these is the association between a high intake of refined simple carbohydrates and an increased risk of Crohn’s disease (187,188). Whether this is a causal association or simply an effect of the disease is unclear. McDonald and Fazio conducted detailed diet-focussed interviews in 71 patients with Crohn’s disease, all of whom had undergone some form of surgery (189). They obtained information about the effects of 32 different foodstuffs, what dietary advice had been given, who had given that advice and the patients’ attitudes to food in general. Healthy controls (n=27) were also interviewed.

Compared to controls, patients who had had an ileostomy reported significantly higher rates of adverse gastrointestinal reactions to carbonated drinks, high in sugar. Only 2/71 patients felt that foods may be responsible for their disease, but the majority thought that dietary factors were important in controlling their symptoms. Stokes described a correlation between the incidence of Crohn's disease and national margarine consumption (190). Shoda *et al* (191) demonstrated a correlation between the rising incidence of Crohn's disease in Japan and rising dietary intake of animal protein, milk protein and total fat, with n-6 polyunsaturated fatty acids a particular association. A diet rich in fruit and vegetables may offer some protection from IBD (187,191,192). Kirsner *et al* dismissed the role of reactions to potential food allergens as non-contributory (193). However, the precise nature of the association between various foods and IBD is difficult to ascertain, not least because all of the published epidemiological studies rely on patient recall of a pre-morbid dietary history. When it is borne in mind that many patients may have experienced symptoms of intestinal disease for several years prior to their eventual diagnosis, the reliability of such histories is cast further into doubt.

2.11.0 Food Antibodies In Inflammatory Bowel Disease

Several authors have described increased levels of circulating antibodies to common foods in the sera of patients with IBD when compared to normal controls. However, their findings are often contradictory. Truelove and co-workers found increased levels of antibodies to cows' milk proteins in patients with ulcerative colitis (194). At one time the presence of detectable antibodies to milk proteins was thought to represent an aetiological link between such proteins and ulcerative colitis. This was backed up, to some degree, by

two studies claiming beneficial effects in patients with ulcerative colitis who followed a milk free diet. This benefit was shown to be independent of possible secondary lactase deficiencies (194,195). Paganelli *et al*, using more sophisticated techniques, showed that these serological findings were not limited to ulcerative colitis, but were also seen in patients with Crohn's disease. They found increased levels of antibodies of the IgG and IgM classes to the cows' milk protein β -lactoglobulin in individuals with IBD (196). No difference was seen in levels of IgA class antibodies in these groups. Similarly, Knoflach *et al* found increased levels of IgG and IgM class antibodies to five major milk proteins (β -lactoglobulin-A, β -lactoglobulin-B, α -lactalbumin, casein and bovine serum albumin) in a group of patients with inflammatory bowel disease compared to levels in normal controls. There was no difference in antibody levels in patients with Crohn's disease compared to those with Ulcerative colitis. IgA antibody levels were not significantly different (197). An earlier study revealed raised titres of antibodies directed against maize in the sera of patients with Ulcerative colitis, Crohn's disease and Coeliac disease (198). These studies span several decades and different techniques such as RIA and ELISA were employed to detect antibodies. In view of this, direct comparisons are unhelpful. The finding by Knoflach of no significant differences in levels of IgA antibodies in sera of IBD patients (197) compared with normal controls is of some interest. It is known that in the normal intestine, the majority of lamina propria plasma cells secrete IgA. IgA is protective against luminal pathogens, does not fix complement and does not excite an intense inflammatory reaction. In contrast, in the setting of inflammatory bowel disease, lamina propria plasma cells have been shown to switch to secreting IgG. This switching is thought to represent terminal differentiation of plasma

cells. IgG can fix complement and is capable of activating natural killer (NK) lymphocytes and phagocytes and hence may contribute to intestinal inflammation.

These studies all measured levels of antibodies in serum, which may not reflect what is happening at the level of the intestinal mucosa. Hoque *et al* studied the levels of IgA antibodies to bacterial cell wall components and to ovalbumin in whole gut lavage fluid from healthy volunteers in Edinburgh and Dhaka. Antibody levels to the core oligosaccharide of bacterial lipopolysaccharide from several Gram-negative species were significantly higher in the Dhaka group, while antibodies to ovalbumin were higher in the Edinburgh group (199). In populations where there is a reduced exposure of the intestinal immune system to enteric pathogens, there may be generation of inappropriate immune responses to dietary antigens. In turn, this may have implications for the development of inappropriate intestinal inflammation.

Observations that patients suffering from inflammatory diseases of the intestine, such as Crohn's disease and Ulcerative colitis, had elevated serum levels of antibodies directed against various common food components were of moderate interest in their own right. However, no obvious differences in the level of expression of antibodies between these two phenotypically different inflammatory bowel diseases had been demonstrated.

In 1988, Main *et al* (81) working in Aberdeen, published the first report of increased levels of circulating antibodies to *Saccharomyces cerevisiae*, an organism found in bakers' and brewers' yeast, in the sera of patients with Crohn's disease but not those with

ulcerative colitis. In their original paper, Crohn's patients were found to have significantly elevated levels of IgG antibodies, detected by ELISA, directed against the organism when compared with results from ulcerative colitis patients and normal controls. They hypothesised that this may indicate a pathological response to this organism, which by its use in the processes of both baking and brewing, was likely to be found in a wide variety of foods central to a modern, Western diet. Here was a possibility, once again, that a hypersensitivity reaction to a common foodstuff might be implicated in IBD, in this case, specifically Crohn's disease.

In a subsequent paper, these authors set out to investigate whether their original findings were exclusive to *S. cerevisiae*, or if they could be extended to other yeast strains. Using similar methodology, they examined the antibody response to 12 commonly used strains of *S. cerevisiae* and to *Candida albicans*, types A and B. These *Candida* strains are the two commonest serotypes found in the human digestive tract, in up to 50% of normal healthy individuals and in a higher proportion of hospital in-patients (200). They found increased levels of serum antibodies to *S. cerevisiae*, in all but one of the 12 strains studied, in Crohn's disease patients, compared with ulcerative colitis patients and normal controls. There was no difference seen in the levels of antibodies expressed against the remaining strain of *S. cerevisiae*, nor in antibodies against either strain of *C. albicans*. The authors inferred from these data that the increased antibody levels to *S. cerevisiae* could not be explained simply on the basis of increased intestinal permeability and hence increased exposure of the intestinal immune system to luminal antigens. This was evidence of a selective antigenic response to *S. cerevisiae* in patients suffering from

Crohn's disease. Further characterisation of the antigenic response and cross reactivity between these 12 strains of *Saccharomyces* and *C. albicans* was performed by cross-absorption testing of antibodies from the serum of a single Crohn's patient. In addition, cross-reaction experiments between *Saccharomyces* and *Candida* were carried out using sera of eight different Crohn's patients. This revealed considerable antigenic heterogeneity between the different strains of *Saccharomyces*. There was also a limited degree of cross reactivity between the different *Saccharomyces* strains and *Candida albicans*. This lends support to the notion that the anti-*Saccharomyces cerevisiae* antibodies (ASCA) were directed against multiple antigens. It is possible that *S. cerevisiae* itself may play an important aetiological role in Crohn's disease, rather than simply acting as an innocent bystander which, by coincidence, shares an epitope with the true, as yet unidentified agent. One potential weakness of these studies was the failure to use another control group with a different type of intestinal disease (e.g. Coeliac disease), or a different inflammatory disease (e.g. Rheumatoid arthritis), to determine whether ASCA positivity is exclusively a feature of Crohn's disease. Later work did reveal increased antibody levels in patients with Coeliac disease but to a lesser degree than that seen in Crohn's (N. Reynolds, personal communication). McKenzie correctly predicted that the antibody response was directed against mannan residues (201), a common antigenic component of both yeast and mycobacterial cell walls. He also hypothesised that the unidentified aetiological agent might be an atypical mycobacterium which supported another theory on the possible aetiology of Crohn's disease (202).

Several other groups reproduced these results but found wide variations in the antigenicity of different strains of *Saccharomyces cerevisiae*, as well as variation in the degree and nature of individual patients' antibody response (203,204). Using *Saccharomyces cerevisiae uvarum* 1 (a strain related to that rogue non-responding twelfth strain in earlier work), Sendid confirmed McKenzie's hypothesis that the ASCA response was indeed directed against mannose sequences in the cell wall. Crude mannan extracts from this strain were used as the coating antigen in an ELISA in which a positive ASCA result was shown to be 64% sensitive and 77% specific for Crohn's disease (205). This same group also undertook a larger study of the relationship between ASCA and Crohn's disease in 1998. In this study, involving a total of 391 patients and controls, they examined the utility of a combination of ASCA and perinuclear anti-neutrophil cytoplasmic antibodies (p-ANCA) for the diagnosis of Crohn's disease and ulcerative colitis. p-ANCA are produced by B-cells in the colonic mucosa and react with a nuclear envelope protein of neutrophils, giving perinuclear "highlighting" on indirect immunofluorescence. They are a useful serological marker of ulcerative colitis but are also found in a subgroup of patients with Crohn's colitis (206). They are not, however, raised against food components. Quinton *et al* (207) looked at ASCA and p-ANCA expression in the sera of 100 Crohn's disease patients, 101 ulcerative colitis patients, 163 healthy members of hospital staff and 27 other disease control patients. This final control group was made up of patients with a variety of diarrhoeal diseases, including microscopic colitis, schistosomiasis, infectious colitis and a single patient with Coeliac disease. Results showed that IgA, IgG or IgM antibody classes of ASCA were positive in 61/100 Crohn's disease and 12/101 ulcerative colitis patients. This gave ASCA a

sensitivity of 61%, a specificity of 88% and a positive predictive value of 89% for Crohn's disease. The authors do not mention in the paper what combination of positive IgA, IgG or IgM results was taken to be an overall positive result. Compared to those with a negative result, ASCA positive Crohn's patients were significantly more likely to be diagnosed at an earlier age (median age 21 years, range 10-59 vs 24 years, range 15-47, $p<0.05$). Of those with a positive ASCA result, 62% had small bowel disease alone, 46% had colonic disease alone and 74% mixed small bowel and colonic disease. There was no relationship between disease activity as measured by Crohn's disease activity index (CDAI) and a positive ASCA result. A multivariate analysis showed that only the presence of small bowel disease was significantly associated with positive ASCA (odds ratio 2.7, 95% CI 1.1-6.7, $p<0.027$). Only one of the healthy control patients had a positive ASCA. However, there were three positive results amongst the non-IBD inflammatory disease control group: one patient with eosinophilic colitis, another with radiation proctitis and the third had sarcoidosis (207).

There has been great commercial interest in the diagnostic potential of ASCA in IBD, especially when used in combination with p-ANCA as an aid to differentiating between Crohn's disease and ulcerative colitis. At least four commercial ELISA kits are now available, all using subtly different components of the *S. cerevisiae* organism as the coating antigen. These are marketed as office based tests to help the clinician distinguish between the two diseases. In another large series, Vermiere *et al* (208), analysed sera from 100 Crohn's disease patients, 100 ulcerative colitis patients, 100 healthy controls and a further 78 non-IBD controls from their local population, based in North Belgium.

This study was a serious attempt to compare the sensitivities, specificities and positive predictive values of all ASCA and pANCA tests in a large cohort of inflammatory bowel disease patients. They critically inspected the results from the four available commercial ASCA ELISA kits. There was wide variation in the sensitivities calculated using different kits, ranging from 41% to 76%. Specificity was inversely related to sensitivity. There was good overall correlation between IgG and IgA ASCA results in all four assays and the interassay receiver operating characteristic (ROC) curves also correlated well. These correlations indicate that there were no differences in the overall performance of all the tests and that the four manufacturers had validated their individual assay cut-off values in similar ways. However, it appeared that different manufacturers had settled on different cut-off values depending on whether a more specific (higher value) or sensitive (lower value) test was required. In contrast to Quinton, Vermiere did not find any relationship between the presence of ASCA, age at onset or site of disease. As seen in the French study, there was no relationship with disease activity. The authors advocate caution in the use and interpretation of these tests and call for standardisation of assays. They conclude that ASCA may be of use in differentiating between Crohn's and ulcerative colitis but is not sufficiently sensitive to be used to screen for the presence of Crohn's disease in a population.

The use of ASCA as a diagnostic tool has been largely motivated by the search for a non-invasive, reliable means of diagnosing IBD, and differentiating between Crohn's disease and Ulcerative colitis, but also by financial concerns. It does, however, mark a shift away from interest in the precise aetiological role of *S. cerevisiae* in Crohn's disease.

This is in spite of the fact that the exact origin of these antibodies and the significance of their presence remains far from clear. Two groups have studied relatives of Crohn's patients and described increased expression of ASCA in the sera of both affected and unaffected family members (209,210). This would support a genetic basis for the expression of these antibodies, but this has yet to be described. Reviewing this subject recently, Rutgeerts reminded us that the gene for ASCA is known to be associated with the tumour necrosis factor microsatellite haplotype A11B4C1D3E3. This suggests that a gene involved in ASCA expression may be present in the human leucocyte antigen (HLA)-region on chromosome 6 (211). It is still not certain whether the finding of a positive ASCA result represents the cause or is simply an effect of Crohn's disease.

2.12.0 Nutritional Therapy Of Inflammatory Bowel Disease

Perhaps the most compelling evidence that dietary components are implicated in inflammatory bowel disease, particularly Crohn's disease, is the effect of dietary therapy in this disease. Even if specific dietary factors may be of secondary importance in the causation of inflammatory bowel disease, nutrition forms a vital part of their treatment, either as an adjunct to other forms of therapy, or indeed, as a primary therapy in its own right.

Gassul *et al* report that up to 85% of patients admitted to hospital for the treatment of a relapse of their IBD had evidence of protein energy malnutrition (212). Gee *et al* described evidence of protein-energy malnutrition in 23% of outpatients with Crohn's disease, judged by biochemical and anthropomorphic data (213). Furthermore, weight

loss, anaemia, deficiencies of vitamins A, D and E, magnesium, calcium and zinc are all well described in patients with Crohn's disease (214-220). Growth failure and delayed sexual development have been reported in between 25 and 40% of children with Crohn's disease (215,218). In spite of knowledge of these problems, recording of basic data in an affected child's medical records, such as height and weight, has been shown to be poor (221). Careful attention to the detection and treatment of specific nutritional deficiencies as well as ensuring an adequate caloric intake forms a vital part of the management of patients with Crohn's disease.

Surgical data from the 1970's supported the use of perioperative feeding with an elemental diet to improve outcomes of surgery in patients with inflammatory bowel disease (222) and prompted the authors to ask whether nutritional measures may have a role to play as a primary therapy in Crohn's disease.

Approximately 30 trials of the use of total parenteral nutrition (TPN) as primary therapy for inflammatory bowel disease have been published and reviewed in detail by Greenberg (223). Most of these trials are retrospective or contain small numbers of patients who differ widely with regard to disease extent and activity. The criteria used for a definition of remission are also variable. There are eight prospective trials involving a total of 170 patients with Crohn's disease (224-231). Patients were treated with "bowel rest" and TPN for between seven and 84 days. Of these 170 patients, 137 (81%) achieved some form of initial clinical remission. However, at follow up, ranging between 12 and 64 months, only 23/102 patients remained in remission. The results are even less striking in

ulcerative colitis. In all, 38 patients were studied in three trials (224,225,230), only 42% achieved an initial remission and only 11% remained in long-term remission over a follow up period of 16 to 64 months. Muller *et al* described initial remission in 25/30 (83%) patients with Crohn's disease treated with TPN for 84 days (231). However, 60% of these had suffered a relapse within two years of treatment, a rate four times higher than that seen in a historical cohort of patients treated surgically in the same institution. The actual efficacy of TPN is likely to be even less than the figures quoted as four of the trials in Crohn's disease (224,226,228,230) and two of the ulcerative colitis studies (224,230) allowed concomitant treatment with corticosteroids or azathioprine, while two studies did not report whether other forms of therapy were employed (225,229).

Interest in using supplemental enteral feeding in Crohn's disease began in the 1960's when low residue, defined formula liquid diets were developed for the United States space programme. In an uncontrolled study in 1973, Voitek (222) *et al* observed that several patients who were taking elemental diet while awaiting surgery experienced an improvement in their symptoms as well as their nutritional status. Unfortunately, no patient avoided surgery in this group. The use of these diets became more widespread on the strength of that report, even though it was an uncontrolled study. Defined formula liquid diets are usually categorised by their nitrogen source and are described as elemental, where dietary nitrogen is a mixture of amino acids, or as oligo-peptide, a mixture of short peptides, or polymeric, where nitrogen is provided as whole protein.

Many possible theories, over and above simply correcting nutritional deficiencies, have been put forward to explain the efficacy of defined-formula diets. These include the exclusion of dietary antigens, thus decreasing the presentation of potentially antigenic stimuli in whole food, alteration of intestinal bacterial flora, decreased entero-hepatic circulation of bile salts (232), decreased synthesis of inflammatory mediators, and provision of specific intestinal nutrients, including glutamine (233). It is increasingly likely that the fat composition of the diet may be more important than the nitrogen source. Diets containing fish oils (omega-3 fatty acids) may compete for substrate in the lipo-oxygenase pathway and so favour the production of a less potent inflammatory mediator (234). Diets which contain low levels of the proinflammatory eicosanoid precursor, linoleic acid, may also have some anti-inflammatory role (235). Defined formula liquid diets have never been tested against placebo as it has been considered unethical to knowingly offer placebo to patients with active Crohn's disease. However, two meta-analyses (236,237) and a recent Cochrane review (238) have examined two main issues in the treatment of active Crohn's disease with enteral nutrition; firstly, the comparative efficacies of enteral nutrition and corticosteroids, and secondly, the comparative efficacies of different formulations of enteral nutrition based upon their nitrogen source.

Griffiths *et al* (237) brought together eight randomised controlled trials of enteral nutrition vs corticosteroids, involving 413 patients. They concluded that enteral nutrition was the inferior treatment (pooled odds ratio, 0.35; 95% confidence interval, 0.23-0.53). Fernandez-Banares (236) considered a total of 16 randomised trials and calculated pooled

odds ratio for all types of enteral nutrition vs corticosteroid therapy as 0.35 (95% CI, 0.23-0.53). This study found peptide-based diets to be significantly inferior to corticosteroids (pooled odds ratio 0.32, 95% CI, 0.20-0.52) with only a trend towards poorer remission rates with elemental diet than with steroids (pooled odds ratio 0.44, 95% CI, 0.17-1.12). Zachos *et al* (238) examined six trials, including two abstracts (239,240) which satisfied their inclusion criteria. The four published papers (241-244) included a total of 253 patients with active Crohn's disease (130 treated with enteral diet and 123 with corticosteroids). Meta-analysis showed a pooled odds ratio of 0.30 in favour of treatment with corticosteroids (95% CI: 0.17, 0.52). The number needed to treat (NNT) with corticosteroids to achieve one remission was four.

These studies confirm that corticosteroid treatment is superior to enteral diet as a primary therapy for achieving initial remission in Crohn's disease. This remains the case even when allowing for the considerable numbers of patients who dropped out from the enteral diet arms because of difficulties tolerating the feed. Most trials using defined-formula enteral diet reflect the situation in clinical practice with a relatively high dropout rate as subjects are unable to tolerate either the feed or the naso-enteral tube. Using a questionnaire administered to 89 patients receiving a total of 147 treatments with enteral diet, Teahon *et al* (245) recorded the reasons given by patients for intolerance to dietary therapy. Sip feeding was tolerated by 85% but the remainder required nasogastric tube feeding. The most frequently cited problem was the sheer volume of fluid consumed daily. Nausea and postural hypotension were common in the first few days of treatment. However, after the first week, only six of 89 patients questioned felt that the taste of the

preparation was a significant problem. It is interesting that in spite of all the perceived problems with dietary therapy, two thirds of patients stated they would choose the same treatment again.

One possible method of action for elemental diet is through a reduction in antigen load delivered to the intestinal lumen by virtue of their lack of whole proteins or peptides. Polymeric diets tend to be more palatable than elemental, but does the presence of whole protein reduce their clinical efficacy?

Griffiths (237) reported on five trials with a total of 134 patients, studying elemental vs non-elemental formulae. No difference in effect was noted (pooled odds ratio 0.87, 95% CI, 0.41-1.83), a finding confirmed by Fernandez-Banares (236) who showed a pooled odds ratio for polymeric vs elemental diets of 1.28 (95% CI, 0.40-4.02). In the Cochrane review, 11 trials (including one abstract) comparing different formulations of enteral diet met predetermined quality criteria for inclusion in the study. Of these, 10 (246-255) compared one or more elemental formulations with a non-elemental formulation while the eleventh trial compared two non-elemental, polymeric diets which differed only by glutamine enrichment in one arm of the trial (256). The primary analysis did not include this study or the abstract. Meta-analysis of the remaining nine studies involving a total of 298 patients with active Crohn's disease (170 patients treated with elemental diet and 128 with non-elemental diet) showed no significant difference in rates of remission between the different formulations of diet [OR 1.15 (95% CI: 0.64, 2.08)].

Elemental and non-elemental diets are equally efficacious in the primary treatment of active Crohn's disease but inferior to treatment with corticosteroids. Patients find the diets difficult to tolerate which leads to a high rate of attrition. This can be avoided to some extent by the use of nasogastric tubes or gastrostomy feeding. The mode of action of enteral feeding is unclear but does seem to involve more than simply improving nutritional status.

The relationship between diet and Crohn's disease activity is likely to be more complex than we think. Although corticosteroids have been shown to be superior to enteral diets in achieving remission, diet may have a more important role to play in maintaining remission. In children, supplemental feeding with enteral diet after induction of remission can help to maintain remission (257). A controlled study from Cambridge showed that, once remission has been achieved, by use of either TPN or elemental diet, patients treated with an exclusion diet, were more likely to remain in remission over six months follow-up than those allowed an unrestricted diet (258). Of the 10 patients in the exclusion diet group, where a single food was reintroduced each day, and subsequently avoided if it provoked symptoms, seven remained in remission at six months, compared to no patient out of 10 who ate an unrestricted diet rich in unrefined carbohydrate fibre. This was followed by a multicentre study in which 136 patients with active Crohn's disease were started on an elemental diet. Of the 93 who persevered with the diet for two weeks, 78 (84%) achieved remission and were randomised to either a 12 week tapered course of prednisolone or an exclusion diet with a similarly "tapered" placebo. After two years of follow-up, the median length of remission in the corticosteroid group was 3.8

months (interquartile range 5.0 months) compared to 7.5 months (15.3) in the diet group. There was a significant difference in relapse rates, adjusted for withdrawals, at two years (79% and 62% for the respective groups [$p=0.048$] (259)).

2.13.0 Conclusions

The general public perceives food allergy and food intolerance as one and the same thing. This is not surprising as the symptoms can be very similar, overlapping with those of IBS. The public also see food allergy and intolerance as a much more common problem than is actually the case. The diagnosis of food allergy, with gastrointestinal manifestations, is largely clinical. It is made more likely by features in the history such as an illness of acute onset, temporally associated with ingestion of the offending foodstuff, a personal or family history of atopic disease, the presence of other, extra-intestinal features of allergy such as dermatitis, wheezing or skin rashes, particularly urticaria. Limited *in vitro* testing using RAST to detect specific IgE class antibodies to implicated foods or *in vivo* testing with skin prick tests help to confirm the diagnosis. Other *in vitro* tests are unlikely to be helpful. The gold standard, for now at least, remains double blind placebo controlled challenges with the offending food but these are time-consuming and unpleasant for the patient. In a significant minority of cases, the symptoms if IBS may be alleviated by avoidance of specific foods. This, however, does not imply a true allergy. Nutritional issues are vitally important in the therapy of IBD, particularly Crohn's disease, as part of a holistic approach to the management of a malnourished, anorexic patient. However, specific nutritional therapy is inferior to

conventional corticosteroid therapy in terms of inducing remission of disease, but may be of more relevance in the maintenance of that remission.

Chapter 3- Methods

Ethical Approval

The Lothian Medicine and Oncology subcommittee of the Ethics in Research Committee granted ethical approval for this study (Appendix 4). A number of ethical issues required consideration prior to commencing this study. In particular, since all the individuals concerned were volunteers, and apart from their reaction to mycoprotein, fit and well, it was vital to ensure that no affected subject would be exposed to any potential health risk by taking part in any aspect of the study. This was particularly relevant with respect to healthy volunteers being subjected to invasive investigations. Any intervention undertaken had to be not only safe, but also relevant to the aims of the study and likely to produce results which were meaningful and from which firm conclusions could be drawn. Furthermore, the degree of intervention proposed had to be appropriate for the likely benefit accruing to either the participants or to the greater good. To expose a healthy volunteer group to interventions falling short of these requirements would not have been ethical. With these points in mind, upper gastrointestinal endoscopy, gastric emptying studies and WGL were proposed as appropriate investigations. Subjects were not being exposed to novel, hitherto undescribed procedures. The utility of endoscopically delivered food challenges have been widely described in the literature, even if they have not gained widespread acceptance (114,116,260), and a similar situation holds true for WGL (84-88,261).

Informed consent was vital and so detailed information was made available to potential participants regarding a description of the nature of each procedure and the risks

associated with each of them. These ranged from the risks of pain and bruising following venepuncture, through to the potential complications of upper gastrointestinal endoscopy under sedation. The use of a “stepped” approach to investigation allowed subjects to choose the level to which they wished to participate. Further written information on more invasive tests was only given if subjects expressed a wish to consider participating. At each step it was made clear that subjects could withdraw at any time. Failure to reply was seen as implicit evidence of a wish to withdraw their consent to participate further, and no further attempts to contact the subject was made, thus respecting their wish for privacy.

All subjects were volunteers and none received any payment in cash or in kind from the investigators for their participation. Those who agreed to travel to Edinburgh had all their travelling and accommodation expenses paid for and were not left out of pocket. Subjects were under no compulsion to take part other than their own desire to participate and were free at any time to withdraw their support for the study.

Our hope was to recruit six patients for each of the invasive studies, and six for WGL if serum studies suggested an immunological basis for the adverse reactions. No formal power calculation was made however, and the decision to recruit six individuals was based on the knowledge that this is the minimum number required in a group to show a statistically significant difference before and after an intervention.

The potential risk of an overwhelming anaphylactic-type reaction to mycoprotein on re-exposure was the most serious risk to be considered. Information was available on the type of initial reaction suffered for each subject who had replied to the questionnaire. To minimise the risk of precipitating a further reaction, it was decided in advance that no subject who had experienced an anaphylactic-type reaction would be exposed to mycoprotein again. Many subjects who had experienced the vomiting reaction described a self-administered mycoprotein challenge which had finally alerted them to the possibility that their symptoms were due to the food. There were no reports of serious adverse events following these challenges, suggesting that further challenge was a safe intervention.

It was also felt that to use a double blind placebo controlled food challenge, which would mean exposure of volunteer subjects to mycoprotein in the expectation of reproducing the vomiting reaction, would not be appropriate. This is because although the challenge would prove (or disprove) that mycoprotein could provoke such a reaction, it would not offer any useful information on the mechanism of the reactions produced. Volunteer subjects would be exposed to the potential harm of mycoprotein, in terms of vomiting and a potential risk of anaphylaxis, without deriving any benefit in terms of elucidating a mechanism for the reaction. Double blind placebo controlled food challenges are very useful in making a diagnosis of food allergy or intolerance but are time consuming, and unpleasant for physicians and patients alike. A similar case can be made for avoiding the use of skin prick testing although a positive test might indicate whether an immediate or delayed type hypersensitivity reaction was implicated in the aetiology of the reaction.

Furthermore, the relevance of skin prick test results to reactions occurring within the gut is controversial. As previously discussed RAST testing offers a non-invasive means of identifying antigen specific-IgE in serum without exposing subjects to a potentially harmful direct antigen challenge. However, the specificity and positive predictive value of RAST is poor, and an excess of false positive results is obtained. As a result of these problems, along with the lack of an available RAST for mycoprotein, it was decided to avoid RAST testing in the present study.

In view of the small, but significant radiation exposure involved in the use of two, back to back isotope gastric emptying studies, only male subjects or females who were no longer of child-bearing age were considered for this aspect of the study. The amount of radiation exposure involved was described to subjects in as near to layman's terms as possible, by reference to radiation exposure in comparison to a standard postero-anterior chest radiograph.

Only subjects who were medically fit to undergo two, back to back upper gastrointestinal endoscopies, under intravenous sedation were considered for this aspect of the study. Subjects were assessed on arrival in Edinburgh to ensure that this was indeed the case. All endoscopies were performed by a single endoscopist, experienced in the procedure and in administration of intravenous sedation. Full facilities were available for resuscitation in the event of an anaphylactic reaction. Continuous oxygen therapy was administered by nasal sponge throughout the procedures and vital signs were monitored by pulse oximetry.

3.1.0 Subject Recruitment and Questionnaire

Individuals who had suffered adverse reactions after eating products manufactured from mycoprotein initially contacted Marlow Foods Ltd. This initial contact took the form of letters or telephone calls from the affected individual, giving a brief description of what had happened and often enquiring whether any other consumer had experienced similar problems. Marlow Foods Ltd. then contacted each individual, asking them to give more details of the problem including what precisely had been consumed and whether alcohol had been taken along with the product. This information was recorded on a *pro forma* in an attempt to create a standard data set.

A total of 140 consecutive cases were prospectively collected by the Customer Relations staff at Marlow Foods Ltd, between January 1997 and December 1997 and their details passed to the Gastrointestinal Unit, Western General Hospital, Edinburgh. These include all “complaints” relating to adverse reactions to mycoprotein received by the company during that 12-month period. In subsequent years, the number of reports of adverse reactions has peaked and remained stable at 200 cases per year. The information contained within these 140 *pro formas* acted as a starting point for the study. However, it soon became apparent that there was considerable variation in the quantity and quality of information available from such a source, with even basic details, such as date of birth and gender, absent in some cases. It was therefore necessary to construct a standard dataset consisting of the relevant demographic and clinical details of each individual case. In order to obtain the information required, a short questionnaire was devised (Appendix 1).

Questionnaires have been widely used in medicine in general, and in the field of allergic disorders in particular, to collect data from large groups of individuals. They are cost efficient and allow investigators to obtain information from the patients' own perspective of the experiences and symptom history. The information obtained from a well designed questionnaire can be useful for documenting the burden of an illness across a given population, or for investigating health service utilisation rates for specific diseases. However, there are a number of general problems associated with the use of questionnaires to obtain clinical information. They allow a degree of subjectivity in terms of a respondent's description of a symptom or event for which it is difficult to control. All questionnaires are subject to recall bias on the part of the respondent, and, by the use of "loaded" or "closed" questions, may be subject to bias in terms of responses favored by the investigator. For example, a respondent's memory of symptoms or events may be patchy, or may be skewed by other personal factors, not always pertinent to the matter under investigation, but nonetheless important to the individual. Similarly, vital details may be forgotten or misinterpreted. Some respondents feel obliged to give an account of events which they feel is expected by the investigator rather than a true representation of what actually happened.

However, all medical consultations revolve around the acquisition of information from the patient by the physician. We have to accept that any information obtained in taking a medical history is subject to the same recall bias as that obtained by means of a questionnaire. Questionnaires tend to replicate the methods employed by physicians to

acquire the necessary information to formulate a diagnosis during a consultation. Interviewer administered questionnaires seek to remove some of the subjectivity associated with self-administered questionnaires but run the risk of introducing further bias by the interviewer.

It was decided that to travel to all affected subjects to administer a structured questionnaire would be time consuming and an inefficient use of resources. Instead, a self-administered questionnaire would be posted to all affected subjects. In order to be a useful tool, a questionnaire has to be written and presented in terms which are easily understood by the respondent, and relatively short to encourage compliance. It must also ask questions which are relevant and valid in relation to the underlying problem. Furthermore, the answers received from respondents should be repeatable or reproducible.

There is no validated questionnaire in existence which explores the prevalence and nature of adverse reactions to mycoprotein. There are a number of validated questionnaires which are pertinent to atopic diseases such as asthma and allergic rhinitis and these have been used extensively in the study of the prevalence of such disorders across the world (262-264). However, meaningful and useful studies of atopic disorders have been performed which utilised short, simple and easily-understood, self-administered questionnaires (265,266). Even with well-validated questionnaires, controversy exists as to whether they alone can adequately measure disease prevalence or give sufficient epidemiological information to comment on clinically important risk factors (267).

Two large population-based studies of food intolerance in the United Kingdom had been published at the time of this study (174,175), one supported by the Medical Research Council. Both used simple, short and easily understood questionnaires which asked “open” questions on perceived food intolerances such as, “do any foods make you ill?”, in addition to enquiring about past or family history of atopic disease in terms of a doctor’s diagnosis of “asthma”, “eczema” or “hayfever”.

The questionnaire devised for this study was based on that employed by Young *et al* (174), in her study of almost 20,000 individuals which asked standard questions directed to a personal or family history of atopy and past medical history. It took the form of a standard medical history, based around the pertinent details in a history suggestive of an allergic reaction. The mycoprotein study involved an alleged reaction to a specific foodstuff, and so the primary aim of the questionnaire was to elicit more detailed information regarding the precise nature of the reactions suffered. The questionnaire, therefore, began with an “open” question, asking for a description of the individual’s own experience of the reaction, in order to avoid the investigator bias which a series of “closed”, symptom-directed questions might introduce.

A subject was included in the atopic grouping if they answered in the affirmative to having a pre-existing diagnosis of “asthma”, “eczema or dermatitis” or “hayfever”. Other details included the subjects’ age, gender and address, as well as details of each General Practitioner (GP) to allow contact to be made for the purpose of collecting blood

samples. Most attention was given to a precise account of the reaction suffered including the temporal relation to ingestion of mycoprotein and the amount of mycoprotein consumed. In addition, details of subjects' past medical history were sought with particular reference to history of allergy or atopic diseases, drug history, family history and details of tobacco and alcohol consumption. The questionnaire also inquired whether individual subjects would agree to have a blood sample taken locally by their own General Practitioner to be sent to Edinburgh for antibody testing (as described in 2.3.11). In a pilot study, 10 individuals, comprising members of medical and non-medical staff within the Gastrointestinal Unit filled in questionnaires to assess for general comments regarding the ease of comprehension and the relevance of questions being asked. No significant changes required to be made to the questionnaire following this exercise.

All affected subjects were assigned a sequential identification number (1-54) as their blood samples arrived in Edinburgh. Whole blood samples were centrifuged and the serum removed and frozen at -20°C in 100 µl aliquots awaiting analysis.

All questionnaires were sent by post to the addresses given in the original Marlow Foods *pro forma*. A stamped, self-addressed envelope was enclosed. In addition a letter was sent to each subjects' GP, containing an explanation of the study, a venepuncture kit and a small honorarium (a cheque for five pounds sterling) for taking and returning the sample.

If no reply was received within a period of four weeks, no further mailing or attempts to contact subjects was made. Since this was entirely a volunteer study, involving subjects who were otherwise fit and well, an initial failure to reply was taken as implicit evidence of a wish not to participate further. All information thus obtained was stored on a Microsoft Access® database.

A stepwise approach to investigation was used. Those subjects who did agree to send a blood sample were approached again by post to ask if they would consider giving up more of their time to travel to Edinburgh to undergo a more invasive investigation, such as upper gastrointestinal endoscopy, whole gut lavage or isotope gastric emptying studies. The procedures themselves along with the rationale for performing them were explained in detail in these letters.

3.2.0 Selection of Normal and Disease Controls

After a full explanation of the nature of the study, fresh serum samples were obtained from healthy laboratory volunteers (n=14), or permission was obtained to use stored, frozen sera. Disease controls were chosen from a storage bank of samples from individuals undergoing investigation of gastrointestinal disorders in the Gastrointestinal Unit, Western General Hospital, Edinburgh. This consists of paired samples of serum and whole gut lavage fluid (WGLF), stored at -70°C. Sera from patients suffering from Crohn's disease (n=22), ulcerative colitis (n=17) and Coeliac disease (n=15) were obtained. As far as possible, attempts were made to match the ages and sex of the

controls to the affected subjects. However, due to the limited availability of stored sera this was often not possible. Details of diagnosis, date of whole gut lavage procedure and results of levels of IgG, α -1 antitrypsin, albumin and haemoglobin are all held on file in a Microsoft Access[®] database. Using this information it was possible to assess the presence or absence of intestinal inflammation in each of the IBD-control patients. Normal ranges for IgG ($<10\mu\text{g/ml}$), albumin ($<26\mu\text{g/ml}$), α -1 antitrypsin ($<19\mu\text{g/ml}$) (89) and haemoglobin ($1\text{-}5\mu\text{g/ml}$) (261) in WGLF had been established previously. Active IBD was defined by the presence of increased levels of these inflammatory markers in whole gut lavage fluid. This has been shown to correlate well with other markers of disease activity such as the Crohn's disease Activity Index (CDAI) (89,261).

3.3.0 Development of an ELISA for measurement of mycoprotein-specific antibodies in serum and Whole Gut Lavage Fluid

3.3.1 Initial preparation of mycoprotein antigen

The ELISA method measures the antigen specific antibody levels to a soluble antigen in body fluids (serum in this case). This method requires insoluble antigens to be made soluble for antibody measurement, often by treating with heat, acid, alkali or by dissolving in organic solvents prior to dissolving in non-organic solvents.

The main constituents of Quorn® are mycoprotein (MP) from the fungus *Fusarium* species ATC 20334 (*Fusarium graminearum*) and egg. Freeze-dried MP and egg albumin were supplied by Marlow Foods Ltd as well as four varieties of the commercial product: Quorn® Pieces, Quorn® Mince, Quorn® Burgers and Quorn® Sausages. As all these Quorn® products are insoluble in water, the antigens had to be processed before use. The antigen preparation and ELISA development was carried out by Dr. Marian Aldhous.

It was decided to try to mimic the procedures which would have occurred in the cooking and eating of these products. A small amount of the freeze-dried MP, uncooked Pieces or uncooked Mince was heated in glass bottles on a hot-plate for approximately 5 minutes. After the addition of 5 ml of distilled water, the foods were 'simmered' for a further 10-15 minutes. Similarly, a small amount of (uncooked) Burger and Sausage was cut into smaller pieces before being heated on the hot-plate in glass bottles for approximately 15 minutes (to mimic grilling). This "cooking" method followed the manufacturer's instructions as near as possible.

The cooked food was allowed to cool slightly and then Pepsin (10µg/ml in 0.1M HCl) was added (to mimic the action of hydrochloric acid and Pepsin in the stomach on the food proteins) and incubated for one hour at 37°C. An incubation period of one hour was chosen as the individuals had indicated that nausea and vomiting usually occurred between one and four hours of eating. It was thought that if the reaction described was immunologically mediated, then the responsible epitopes were likely to be unmasked

during this time period. The antigen preparation was then neutralised with alkali and the supernatant was collected for use in the ELISA. An insoluble residue of Quorn[®] products was left at the end of this procedure. This was discarded and was not assayed further in any way. Each batch of antigen was stored at -20°C in 150µl aliquots. One aliquot of antigen was used for each plate.

3.3.2 Preparation of egg antigen

A commercial preparation of egg protein, identical to that used in the manufacture of Quorn[®], was supplied by Marlow Foods for use in another ELISA. This preparation is a mixture of two common egg antigens, ovalbumin and ovomucoid. The powdered egg antigen was soluble in water and so did not require any of the preparation mentioned above.

3.3.3 Quality control- inter-batch variation

In order to look at variation between batches of antigen preparations, batches of all antigen preparations were made up on three separate days using the same methods. On days one and two, 150 µl aliquots of antigen were frozen at -20°C, and on day three, antigen was prepared freshly. The thawed antigen preparations from days one and two and the fresh antigen were all used on day three to coat six 96-well Immulon-2[®] (Dynex Laboratories) ELISA plates. All six plates were run in parallel using standard sera in the ELISA procedure described below. The frozen/thawed antigen and fresh antigen preparations were found to give similar results of antigen recovery and so variation was thought to be negligible. It was also reasonable to use frozen antigen, thawed as required

on the day of each batch of experiments. The same batch of MP antigen was used for all the subsequent antibody assays on subjects, normal and disease controls.

3.3.4 ELISA Method For Specific IgG And IgA Antibodies In Serum And Whole Gut Lavage Fluid

The development of the ELISA method for these antigens was an adaptation of an established method for measurement of antibodies to Ovalbumin (268).

Investigation of humoral mucosal immunity presents significant practical and logistical difficulties. From the outset, it was decided to explore the possibility of a humorally mediated immunological reaction in affected subjects by measuring levels of specific IgA, IgG and IgE antibodies to mycoprotein and egg in serum. In spite of the potential problems with a study based on serum measurements alone, there were several reasons for choosing serum as the initial medium. Not least of these was the logistical problem of obtaining samples from subjects scattered throughout the UK. Serum could be more easily obtained by the subjects' GP and posted to Edinburgh for assay. In the first instance serum was seen as the most convenient fluid to obtain and assay.

It is potentially misleading to use information obtained from serum or saliva as representative of events at the mucosal level. Previous work performed in our laboratory did show a poor correlation between levels of systemic and mucosal antibodies to food antigens (88) and to *Klebsiella pneumonia* (87), illustrating the potentially misleading results obtained from studies which relied on measurement of systemic antibodies alone.

For example, in comparison to normal controls, patients with Crohn's disease and ankylosing spondylitis, demonstrated significantly higher levels of serum IgA class antibodies to *Klebsiella*, while levels of WGLF IgA antibodies to *Klebsiella* were similar in the three groups (87). In coeliac disease, there was no correlation between systemic and mucosal humoral immune responses to the food antigens gliadin, β -lactoglobulin and ovalbumin (84). These studies highlight the need for more direct study of the mucosal responses to luminal antigens in definitive, descriptive studies of intestinal immunity, rather than relying on studies of serum antibodies alone.

There are circumstances where the extrapolation of serum results does appear to be representative of mucosal events, such as the study of specific IgA-secreting B-cells following enteric infection or immunisation (82). In the clinical arena, serum levels of antibody to gliadin have been extensively used in the interpretation of mucosal events in coeliac disease, although their use had been superseded by more sensitive and specific assays of anti-endomysium and anti-tissue transglutaminase antibodies. Anti-gliadin antibodies of IgA and IgG class have been used in the diagnosis of gluten sensitive enteropathy and subsequently in the monitoring of patients' compliance with a gluten free diet.

However, in the same laboratory, studies on systemic and mucosal responses to food antigens in 15 normal healthy controls and 54 patients with Crohn's disease, ulcerative colitis and coeliac disease, Ferguson *et al* found very few instances where mucosal antibodies were present in the absence of serum antibodies (88). The present study

involved otherwise healthy volunteers, all of whom would either have to travel to Edinburgh to undergo WGL, or have the procedure performed nearer to their home by one of the investigators travelling around the country. Neither of these options was ideal in practical terms, firstly, as subjects would be reluctant to travel and secondly since the WGL procedure should be performed under the close supervision of an experienced practitioner in the hospital setting, which would require permission to use space within a local hospital. Furthermore, proper processing of WGLF, especially the addition of proteases to prevent degradation of immunoglobulins is crucial if the samples obtained are to reflect accurately levels of antibodies and immunoglobulins in intestinal secretions. Any significant delay in the addition of proteases, such as might occur if samples are obtained in settings outwith the Gastrointestinal Unit, has been shown to lead to significant loss of immunoglobulin content in WGLF (85).

Finally, although previous work does demonstrate dissociation of the systemic and mucosal immune responses to luminal antigens, these responses are not by implication completely independent. For example, the increased levels of IgG seen in active inflammatory bowel disease may be partly explained by isotype switching of B-cells and plasma cells in the lamina propria in response to inflammation, but are also derived in part from spillage of serum IgG from the vascular compartment into the lumen across an increasingly permeable mucosal barrier. The issue of the degree to which plasma leakage contributes to the presence of IgG in WGLF has not been adequately resolved although it is quite certainly not the only source of the IgG detected. By the same token, increased serum levels of IgA antibodies to food proteins may be seen in coeliac patients, which

after a period on gluten free diet return to levels seen in normal controls. This may be partly derived from spillage across the inflamed mucosa in the opposite direction, into the vascular compartment.

For these reasons it was decided that it would be reasonable to use measurements of levels of serum antibodies to mycoprotein and egg as a “screen” to look for evidence of an immunological reaction. WGL was reserved as a second line investigation to be used if these initial results in serum suggested an immunological basis to the reaction. In the original outline of investigation presented to the ethics committee, it was stated that WGL would only be performed if any possible humoral immune response was discovered in initial testing performed on serum. In this way it would be possible to minimise the need for travel and avoid invasive investigations in this group of subjects.

In spite of the lack of intestinal intubation, WGL is not a simple procedure and many patients find it unpleasant. To describe WGL as “non-invasive” is to ignore the considerable distress suffered by a significant minority of patients who have experienced it. The volume required to obtain “clear” samples is variable but can be far in excess of 4 litres and the procedure can go on for hours. Complaints of nausea, vomiting, abdominal bloating and cramps are common and indeed, anecdotally, many patients who have experienced WGL are quite definite that they will never undergo the procedure again, after finding it more traumatic than the subsequent barium study or colonoscopy.

In summary, it was accepted that in view of the nature of this study, involving otherwise healthy subjects scattered throughout the UK, collection of WGLF from each subject would present enormous logistical difficulties. Any attempt to collect samples on such a scale would have been impractical. WGLF samples might not be adequately processed and large numbers of otherwise healthy individuals would be exposed to a comparatively invasive, uncomfortable procedure. Previous data from our laboratory demonstrated a very low yield of positive mucosal antibodies in WGLF in inflammatory bowel disease patients with no corresponding serum antibodies and no positive mucosal antibodies in healthy individuals without corresponding serum antibodies. In the specific circumstances of this study, it was felt reasonable to undertake a study for mucosal antibodies in WGLF in a subset of subjects only if there was compelling evidence for an immunological reaction from the initial serum studies.

3.3.5 Choice Of Conditions

Dr Marian Aldhous performed the preliminary sets of ELISA experiments to determine the best conditions for the assay. These experiments also determined the optimum choice of ELISA plate, dilution of antigens for plate coating, dilution of samples and the need for blocking the non-specific binding of serum proteins to the ELISA plates. Details of the assay procedure are given in section 3.3.11.

3.3.6 Choice Of Plates

Immulon-2[®] “High-binding” ELISA plates (Dynex Laboratories) were used, as opposed to the Immulon-1[®] “Medium/Low-binding” ELISA plates. These 96-well, plastic plates

are often used when binding of a potential antigen is anticipated to be weak. They are thought to have a greater affinity for antigen and so may enhance binding of proteins. On comparison of the plates, Immulon-2[®] plates were found to be more efficient at binding the antigen.

3.3.7 Coating

The optimal dilution of all antigens for coating was found to be 1:100, except egg albumin, which was used at a concentration of 10µg/ml.

3.3.8 Blocking

Initial pilot experiments showed more non-specific binding of serum proteins to the plastic wells than expected, but this was eradicated when a blocking protein in the form of human haemoglobin was added to each well. Human haemoglobin was chosen as it did not interfere with the assay and was unlikely to be present as a normal ingredient in the foods.

3.3.9 Addition of Samples (Choice of Dilutions)

Details of sample dilutions are given in the individual section dealing with each assay.

3.3.10 Choice Of Standards And Quality Controls

Sera from patients being investigated for gastrointestinal symptoms in the Western General Hospital, Edinburgh, were stored in Gastrointestinal Unit Laboratory. These were screened to find individuals demonstrating consistently high levels of specific-

antibody to the food proteins being tested. Progressive dilutions of these sera were then used on each plate, in each batch of experiments to construct a standard curve against which all other sera would be tested. In addition, sera were chosen from two other individuals who showed consistently high levels of specific antibody to the egg and various mycoprotein preparations. These were used on each plate in each batch of experiments as quality controls to allow a measure of the degree of variability of the assay between plates and from day to day. The “standard” serum, against which all other samples would be compared, and two quality controls were all obtained from patients suffering from Coeliac Disease, already adhering to a gluten-free diet. In accordance with local policy, the nature of the study was explained to the individuals concerned and written permission was obtained both for testing their serum for Human Immunodeficiency Virus (HIV), Hepatitis B and Hepatitis C viruses (HBV and HCV) prior to use for the assay.

3.3.11 Sandwich ELISA Procedure

The Quorn[®] antigen-preparations were diluted in coating buffer (bicarbonate/carbonate buffer (pH 9.6), Sigma Laboratories) at a dilution of 1:100. The egg albumin was used at a concentration of 10µg/ml. One plate was used for each antigen. After incubation for 5 hours at 22°C in a moist box, the plates were washed three times with ELISA wash (0.9% Saline (Baxter's) containing 0.05% Tween 20 (Sigma). The plates were blocked with extra protein (ELISA wash containing 10µg/ml human haemoglobin (Sigma) and 1% adult bovine serum (Scottish Antibody Production Unit)) to stop non-specific binding of irrelevant proteins to the plastic. The plates were then incubated for 1 hour at 37°C. The

plates were washed again. Serum samples were diluted 1:100 in ELISA diluent (ELISA wash containing 1% adult bovine serum) and added to the plates in duplicate, against a "standard" and quality control (QC) samples (as described in 3.3.10). The plates were incubated overnight at 4°C. The amount of specific antibody in serum was detected using an Alkaline Phosphatase-conjugated antibody, specific for either IgA or IgG, diluted in ELISA diluent at 1:1000 and incubated at 22°C for 5 hours. The assay was developed using a colourless substrate for the alkaline phosphatase enzyme (p-nitrophenyl phosphate dissolved in diethanolamine buffer). This chemical is cleaved by alkaline phosphatase to produce a yellow-coloured product (p-nitrophenol) which can be measured by optical density on a spectrophotometer (ELISA reader) at 405 nm wavelength. The amount of colour produced is directly proportional to the amount of antibody present in the serum sample assessed and the results were calculated from the standard curve. Samples that were found to have high levels (above the top standard) of MP-specific IgA antibodies in serum, were re-assayed, double-diluting the samples from 1:100, 1:200 to 1:12800 so that at a higher dilution a value within the standard curve range was obtained and an accurate titre could be determined.

3.3.12 ELISA Method For Specific IgE Antibodies In Serum.

This was based on the same method, but was only used for MP. The main difference in the assay is that an Alkaline Phosphatase-conjugated antibody specific for IgE was used at a dilution of 1:500. As no standard was found with IgE-specific antibodies for MP, the patient samples assayed were used at a dilution of 1:2 and titrated down the plate. The

results were compared between patients and the values are expressed as the optical density (OD) at a 1:2 dilution.

3.3.13 Measurement of total IgE in serum of affected subjects

Total IgE level in 54 affected subjects was measured using a standard ELISA assay at the Department of Clinical Immunology, Blood Transfusion Service, Royal Infirmary, Edinburgh. These were compared with previously calculated normal levels from the local population.

3.4.0 ELISA Method For Detection Of Specific Mycotoxins in serum.

These assays were performed with the help of Dr. Marian Aldhous. ELISA assays for the toxins Zearalenone (ZL), Deoxynivalenol (DON) and Diacety-scirpenol (DAS) were also set up. These toxins are insoluble in aqueous solution, so they were first dissolved in organic solvents, according to the manufacturer's recommendation, and then diluted with the coating buffer. DAS and DON were dissolved in a small volume of chloroform followed by ethanol and then subsequently in ELISA diluent, while ZL was dissolved in ethanol and subsequently in ELISA diluent. Immulon-2[®] plates were used, one plate per patient. For coating, the first 2 columns contained the MP antigen at 1:100 and then titrated out down the plate. Then, each of the next 6 columns contained a toxin: DAS, DON and ZL and titrated out from 1µg/ml down the plate. The next 2 columns contained a cocktail of all three toxins together and the last 2 columns contained the diluent alone,

to check that the small amount of organic solvent present did not interfere with the assay. The plates were incubated and blocked as previously described in section 3.3.11. The patient samples were added to the whole plate at 1:100 dilution and incubated and detected as described in section 3.3.11.

3.5.0 Competition ELISA Method For Mycotoxins, Mycoprotein & Egg

Immulon-2[®] plates were coated with the MP antigen preparation at a dilution of 1:100 in coating buffer. After incubation, the plates were washed and blocked as previously described. One patient serum sample was used for each plate. The toxins used as competing antigens are not soluble in water and were prepared again as in section 3.4.0. For the assay, in the first two columns (duplicates) the patient serum was diluted at 1:100 and titrated out down the plate, to create a "standard curve" for each patient. For the rest of the plate the serum was used at 1:100. In the next two columns, DAS was added to the diluted serum as a competing antigen (at a concentration of 1µg/ml). The competing antigen (i.e. DAS) was titrated out down the plate. Likewise, in the next two columns DON was added to the diluted serum (at a concentration of 1µg/ml) and titrated out down the plate. In the next two columns ZL (also at a concentration of 1µg/ml) was added to the serum and titrated out down the plate. In the next two columns the competing antigen was the egg albumin (used at a concentration of 5µg/ml), to determine whether there was a preferential binding of the antibodies to either of the two components of Quorn[®]. In the last two columns the competing antigen was the MP itself (at a dilution of 1:10) to show that it can bind the antibodies in solution and not just when it is attached to a solid

surface. The plates were incubated overnight as before. After washing the plates, the amount of specific antibody bound to the antigen on the plate, i.e. which had not been bound by the competing antigen, was detected as previously described, using the IgA or IgG specific Alkaline Phosphatase-conjugated antibodies. The assay was developed as previously described and the results were compared with the curve produced from the serum with no competing antigen i.e. the highest value of the curve without competition was taken to be the value of 0% inhibition and all other values were compared accordingly. The results were expressed as the percentage inhibition of binding by the competing antigen.

3.6.0 Competition ELISA for *Saccharomyces cerevisiae*

S. cerevisiae is the organism present in baker's and brewer's yeast and hence is a ubiquitous dietary protein and potential antigen. As previously stated, high levels of specific anti-*Saccharomyces cerevisiae* IgG and IgA antibodies have been reported in the sera of patients with inflammatory bowel disease, and in Crohn's disease in particular (81,201). In the past, it has been suggested that *S. cerevisiae* may play a role in the aetiology of Crohn's Disease, either as a causative agent, or perhaps more likely, by sharing antigenic epitopes with another, as yet unidentified causative agent.

Competition assays using *S. cerevisiae* as the competing antigen were performed to see if there was any cross-reactivity between *Fusarium* species ATC 20334 and this ubiquitous dietary protein. These assays were carried out in the same way as described above, with the competing antigen added at 100µg/ml.

3.7.0 Endoscopic & Histological Appearance of Gastric Mucosa Pre & Post Mycoprotein Challenge

Three subjects, (subjects 14, 35 and 47) who had experienced the idiosyncratic vomiting reaction consented to travel to Edinburgh and undergo two upper gastrointestinal endoscopies under sedation with intravenous midazolam. All subjects were thought to be medically fit to undergo such procedures. All six procedures were performed by a single endoscopist (myself). Subjects were given supplemental oxygen during each procedure and monitored in the standard manner employed in our unit by continuous pulse oximetry throughout the procedure.

Following an overnight fast, upper gastrointestinal endoscopy was performed using an Olympus Q-200 video endoscope. The oesophagus, stomach and duodenum as far as its second part were carefully examined and specific comment made on the presence or absence of gross macroscopic pathology such as hiatus hernia, mucosal erosions or ulceration and evidence of past ulceration. Endoscopic photographs were taken, along with a gastric antral biopsy for rapid urease test (CLO[®] test) to detect the presence of *Helicobacter pylori* infection, and two gastric antral and two gastric body biopsies for conventional histological staining. When the subject regained consciousness an oral mycoprotein challenge in the form of 5g (cooked weight) Quorn[®] Sausage was given. This dose was chosen to produce an attenuated version of subjects' original symptoms without producing undue distress and was derived from information obtained from replies to our original questionnaire. Within the constraints of a busy endoscopy list, we attempted to standardise the period of time between the mycoprotein challenge and the

second endoscopy. Three hours later the procedure was repeated looking for macroscopic evidence of acute inflammation, with particular reference to areas of mucosal erythema or petechial haemorrhage, and repeating all biopsies except the rapid urease test. All biopsies were examined and reported by a single Consultant Histopathologist who was blinded as to the subjects' details, the dates of the procedures and the presence or absence of a mycoprotein challenge.

3.8.0 Isotope Gastric Emptying Studies Pre & Post Oral Mycoprotein Challenge

One subject (subject 27) agreed to attend for isotope gastric emptying study pre and post oral mycoprotein challenge. Similar, paired experiments were carried out on a volunteer control subject who had previously consumed mycoprotein but had never experienced any adverse reaction.

Following a 10 hour fast, at 5pm on day one, each subject was given a standard test meal of 150 ml of pasteurised egg mixed with a radio-pharmaceutical in the form of ^{99m}Techetium-labelled macro-aggregated human serum albumin (12 MBq of radio-activity) microwaved to scrambled egg texture. This was washed down with 20-30ml of water. Time zero was taken as the point at which all the test meal had been swallowed. The subjects were positioned in the anterior and posterior erect position and the amount of radio-active isotope remaining in the stomach was measured using a gamma camera (Elscint[®], General Electric) at 0, 5, 10,15, 30, 45, 60, 90 and 120 minutes. Views were acquired for 60 seconds at the "General" setting with frame size 128, 1.0 zoom. The rate

of solid phase gastric emptying was calculated using a standard software package (Elgems, General Electric). Subjects were allowed an evening meal and a light breakfast the following morning. They then had to begin another 10 hour fast, so that at 5pm on day two, the procedure could be repeated with the same test meal but with the addition of a mycoprotein challenge in the form of 5 g (cooked weight of Quorn[®] sausage).

All isotope gastric emptying studies were reported by a single Consultant Radiologist with an interest in Nuclear Medicine who was blinded as to the subjects' details, the dates of the procedures and the presence or absence of a mycoprotein challenge.

3.9.0 Whole Gut Lavage

One affected subject (subject 5) also underwent a Whole Gut Lavage (WGL) procedure, under supervision in the Gastrointestinal Laboratory, Western General Hospital, Edinburgh. This technique has been well described by Ferguson *et al* (86,89,261) and involves drinking 2000-4000ml of polyethylene glycol (PEG) electrolyte solution at a steady rate of 100ml per hour, which has a powerful laxative effect, until the faecal effluent runs clear. The PEG solution is ingested and moves through the gut at a steady rate of about 20ml/min, collecting all the substances secreted into the lumen. Once the faecal effluent runs clear, it is effectively a "steady-state" gut perfusate. The effluent is then filtered and can be stored at -70°C awaiting further analysis.

3.10.0 Statistics

Comparisons between antibody levels in the different groups of subjects and controls were made using the Kruskal-Wallis test, the non-parametric equivalent of the ANOVA test. This is the most appropriate test to identify significant differences between three or more groups and is particularly useful when data includes outlier values. The Mann-Whitney-U test was used where comparisons between two groups of data were required.

Chapter 4- Results

4.1.0 Questionnaire Results

4.1.1 Initial respondents and non-respondents

Respondents

A total of 90/140 subjects replied to the questionnaire giving a 64% response rate. Of these, 44.4% were male and 55.6% were female. The mean age of this group was 47 years with a range between five and 88 years old. Subjects aged less than 16 years were excluded from further testing. Of these 90 subjects, 81 agreed to consider further tests to explore the cause of their reaction. Of the remaining nine subjects, five declined any further testing, three made no comment and one declined as she was now living in Australia.

Replies were received from all over the United Kingdom, with no obvious geographical clustering of cases, with the exception of two subjects who lived within two miles of one another in Mickleover, in the Derby area. There was no preponderance of urban or rural addresses.

Fifty-four percent (49/90) of respondents stated that they had never experienced an allergic reaction prior to this episode. However, of these 49 individuals, 12 (24%) also stated that they had suffered from an atopic condition at some point in their lives. In addition, on specific questioning, four (8%) gave information on previous reactions to drugs including penicillin, tetracycline and aspirin, and three (6%) stated they had suffered from nickel allergy.

The remaining 41/90 (46%) of subjects *had* experienced an allergic reaction of some kind in the past but only 23/41 actually gave an example of the offending substance. In total, 10/23 (43%) had reacted to another foodstuff, such as mushrooms (2/23), shellfish (2/23), peanut (1/23), and egg white (1/23). Contact allergy to nickel jewellery occurred in 7/23 (30%), with reactions to animal dander and to drugs accounting for the remaining 27%.

The questionnaire deliberately made the distinction between a history of “allergy” and that of atopic conditions, with asthma, eczema and hay-fever mentioned specifically. Of 90 initial respondents, 43 (48%) described a personal history of such atopic conditions. In 37/43 (86%) of cases, these problems were current but 14% of subjects had “grown out” of a childhood atopic tendency. One subject was taking regular medication, oral Prednisolone, for severe asthma. In spite of specific enquiry about medication, no other subject gave information on use of inhaled corticosteroids or bronchodilators.

With the exception of atopic disease, a history of any other past medical problems was unusual in this group. One subject suffered from juvenile chronic arthritis, another from coronary heart disease, another had been an intravenous drug abuser in the past, one suffered from chronic fatigue syndrome and two had chronic skin conditions (psoriasis and acne rosacea). Two out of 90 subjects gave a significant history of dyspeptic symptoms over and above those suffered after consuming mycoprotein, one was awaiting upper gastrointestinal endoscopy to investigate this further. One subject had symptoms of irritable bowel syndrome affecting the lower gastrointestinal tract. No other subject had any history of significant gastrointestinal disease. Atopic history is included in Figure

4.1 as a pointer towards previous allergic type reactions. Figure 4.2 shows that the majority (81%) of subjects who described vomiting had reacted on their first exposure to mycoprotein compared with only a third (36%) of those who described a non-vomiting reaction

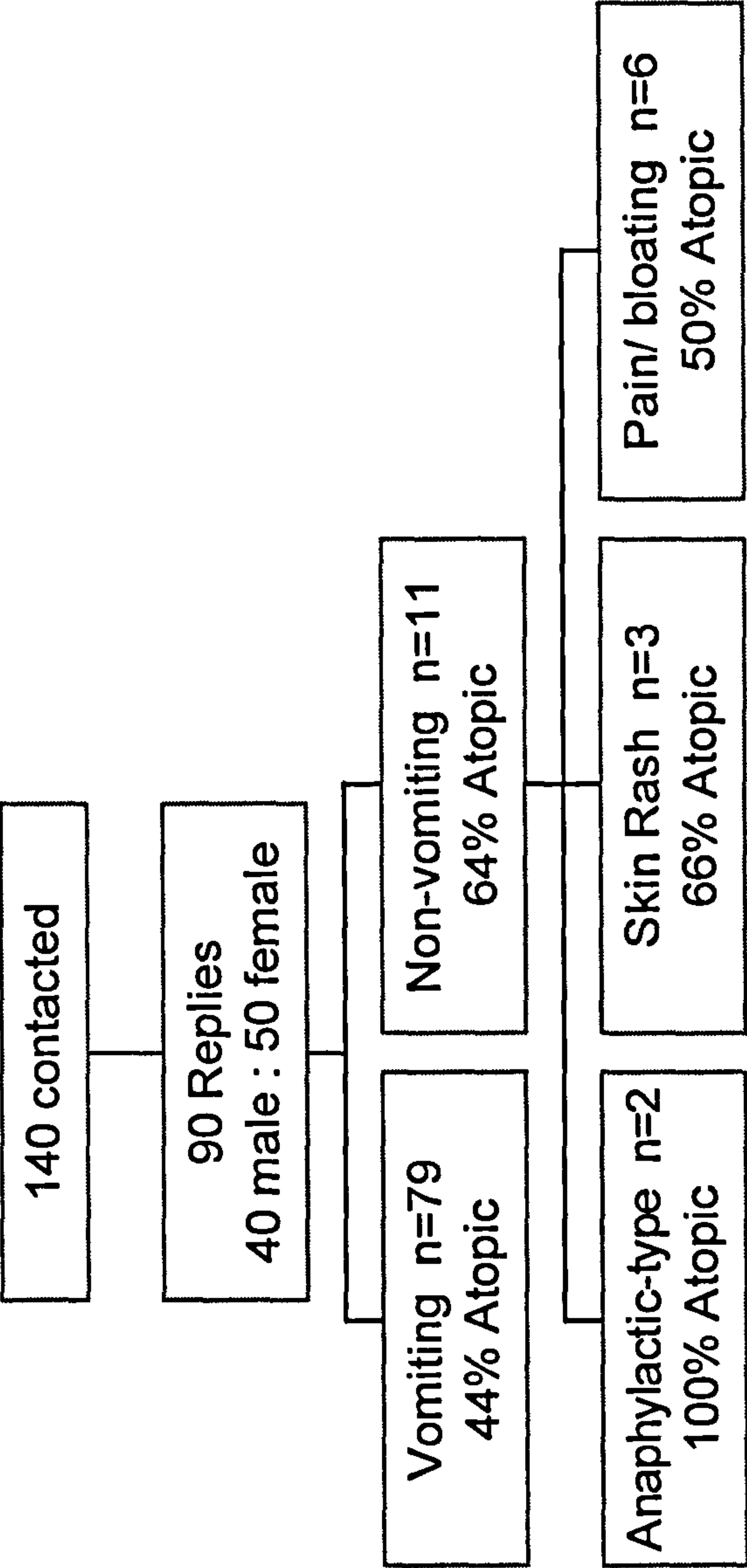
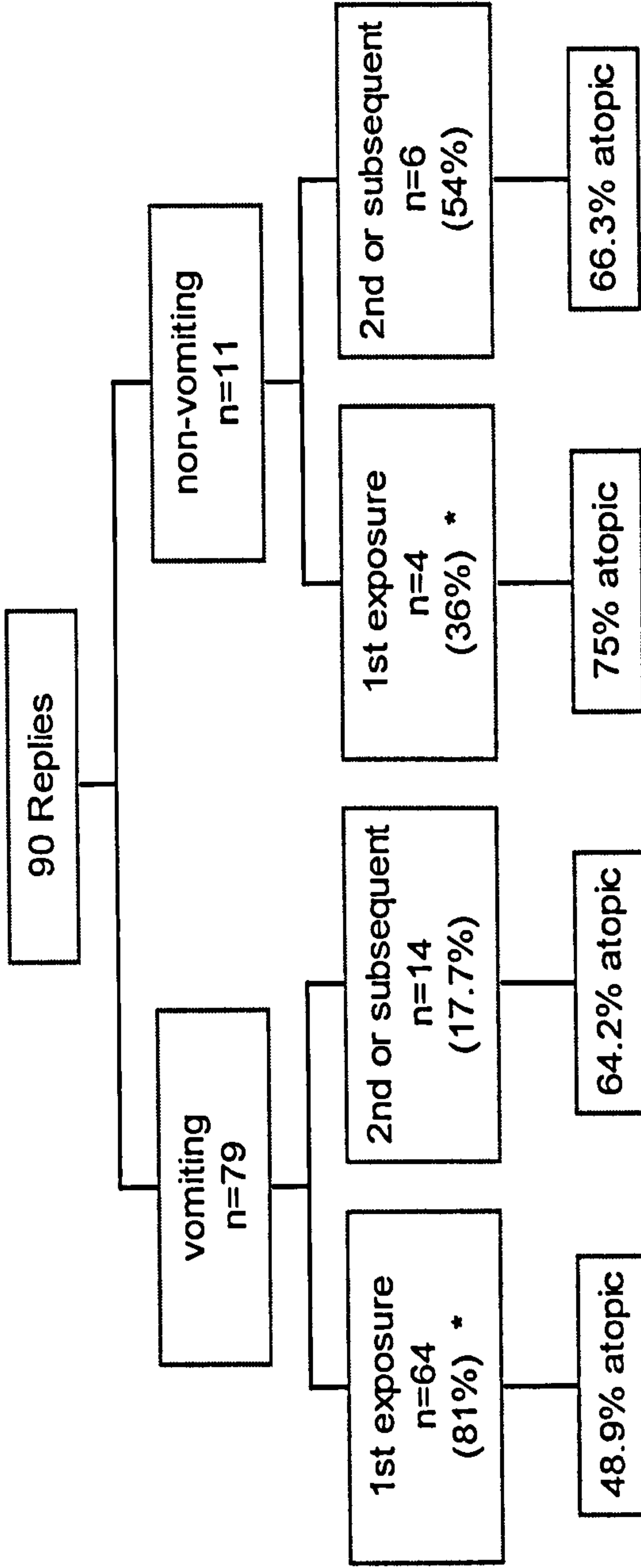


Figure 4.1 Breakdown of subjects according to the type of reaction described



* One subject in each group did not state whether they reacted on first or on subsequent exposure to mycoprotein

Figure 4.2

Non respondents

50/140 subjects declined to respond to the postal questionnaire. As previously stated, an initial failure to reply was taken as implicit evidence of a decision not to participate and the subjects' privacy was respected thereafter. One of the primary aims of setting the questionnaire was to address the paucity of information on those affected by adverse reactions to mycoprotein and to produce a standard dataset of information which could be used for comparison. Because none of the non-responders provided any additional information the demographic information on this group is patchy with many omissions and some apparent incongruities of data. For example there is no information on age in the initial *pro-formas* issued by Marlow Foods Ltd, but 36/50 questionnaire non-respondents have provided postcode information. Therefore, only limited comparisons can be made between initial responder and non-responder groups according to the information available.

However, in spite of this, some comparisons can be made. There was a much higher proportion of females in the questionnaire non-responder group at 86% (vs 55.6% females in the responder group). Overall, the relative proportions of subjects suffering nausea or vomiting (42/50), skin rashes (3/50) or respiratory difficulties (3/50) after ingestion of mycoprotein is remarkably similar in non-responders compared with the responder group. Of the 36 non-responders on whom postcodes were available, 21 were urban addresses and 15 rural. Of the 31 who commented on whether adverse reactions had occurred on their first, or subsequent, exposure to mycoprotein, 20 reacted on first exposure.

Of the non-responders, 10/50 had alluded to a previous allergy when first contacting the company. Previous allergies mentioned specifically included sensitivities to mushroom, penicillin, seafood, dairy and nickel. The remaining 40/50 non-responders had made no comment so no conclusion can be drawn from their silence. No useful comment can be made on the presence of atopic disease since only 5/50 volunteered a previous history of atopy, with 9/50 denying any atopic tendency and the remaining 36/50 making no comment.

In summary, from the incomplete dataset consisting of the limited information available from the initial Marlow Foods Ltd *pro-forma*, it can be seen that there was a much greater percentage of females in the questionnaire non-responder group compared to the responders. Furthermore, the relative proportions of those suffering vomiting, skin rash or respiratory type symptoms after ingestion of mycoprotein was similar in both groups. As with the questionnaire respondents, there was no preponderance of urban or rural postcodes. Unfortunately, there is insufficient data to make any other useful comment or comparison on age, previous history of allergy or presence of atopic disease or whether reactions occurred on first or subsequent exposure to mycoprotein between the two groups.

4.1.2 Respondents Sending Serum For Analysis

A total of 54/90 (60%) of the initial group of respondents sent serum samples for analysis. Of this group, only 10 indicated that they would be willing to consider travelling to Edinburgh to have one of the more invasive procedures involving re-challenge with mycoprotein. The subgroup of 54 subjects was representative of the initial sample, as 39% were male and 61% female compared with 44.4% and 55.6% respectively in the whole group. The mean age was 50 years (47 years in the whole group) with a range from 16 to 82 years old. One subject did not complete the questionnaire fully and only limited information is available regarding past history of allergic or atopic disease.

Of this subgroup of subjects, 31/54 (57%) stated that they had never experienced any allergic reactions prior to this episode. However, once again, 6/31 (19%) described a history of atopic disease and, on specific questioning, 3/31 (10%) described reactions to drugs and 1/31 (3%) to nickel.

A previous allergic reaction was described in 22/54 (41%) of subjects. Reactions to another foodstuff were described in 5/22 (23%) of cases. Contact allergy to nickel was reported in 4/22 (18%), reactions to a prescribed drug in 4/22 (18%) and to a wasp sting in one (5%). A personal history of atopic disease was reported by 16/22 (73%) of subjects describing a previous reaction, of which 14/16 (88%) were current problems and 12% had been childhood diagnoses.

Again, for this subgroup, 47/54 (87%) presented with the vomiting reaction, 79% reacting after their first exposure to mycoprotein. Of these individuals, 32/47 (68%) described vomiting as their sole symptom, 9/47 (19%) described vomiting with abdominal pain or bloating, and 6/47 (13%) described vomiting with diarrhoea.

Of the remaining seven subjects presenting with a non-vomiting reaction, five (9%) presented with abdominal bloating alone, two (4%) with anaphylaxis (involving swelling of the lips or mouth with respiratory difficulties, but no cardiovascular collapse) and one (2%) with an urticarial skin rash. Only four (57%) of the non-vomiting group experienced a reaction following the first exposure to mycoprotein.

In contrast to the larger group, the prevalence of atopic disease was broadly similar in both the vomiting and non-vomiting group (48% and 42% respectively).

4.2.0 Details Of Affected Subjects, Normal & Disease Controls

Table 4.1 shows the gender mix and mean age (standard deviation) of those affected subjects who sent blood for analysis along with normal and disease controls.

Of 22 Crohn’s disease (CD) controls, four had disease confined to the ileum alone, four had disease of the colon alone, eight had disease involving both ileum and colon, two had more proximal small bowel disease and one had isolated peri-anal disease. There was no information obtainable on disease extent in the remaining three patients. Active disease (as defined in section 3.2.0) was present in 12/18 Crohn’s disease patients. Active mucosal inflammation or blood loss was present in 12/18 patients with Ulcerative Colitis

(UC) at the time of this study. Of the 15 patients with Coeliac disease, all claimed to be adhering to a strict gluten free diet at the time of anti-endomysium antibody testing. However, this may not be strictly true, as all Coeliac patients included in this study were positive for anti-endomysium antibody, suggesting recent exposure to gluten.

	<i>n</i>	Male : Female	Mean Age (SD)
Subjects	54	20:34	50 (16)
Normal controls	14	8:7	35 (9)
Coeliac controls	15	3:12	45 (15)
Crohn's controls	22	5:17	41 (12)
UC controls	17	10:8	41 (18)

Table 4.1 Age and gender in each group tested

4.3.0 ELISAs For IgA & IgG In Serum

Assays were performed for IgA and IgG antibodies to mycoprotein itself (MP), a range of products containing mycoprotein (Quorn[®] Mince, Pieces, Burgers and Sausages) and also to egg albumin. The results for levels of antibodies to the purified, freeze dried mycoprotein were found to be more reproducible than the values seen for the individual types of Quorn[®] product. As the Quorn[®] products contain other ingredients which may themselves have caused a serological reaction, only the results for mycoprotein and egg albumin (ie the individual constituents of Quorn[®]) are presented.

Results for IgG and IgA antibodies in serum are presented in the form of scatter plots with a horizontal line to represent the median value. All results are expressed in arbitrary units derived from the same standard in each assay.

4.3.1 Serum IgA & IgG Antibodies to Mycoprotein in Subjects and Controls

Looking first at specific IgA antibodies to mycoprotein (figure 4.3), the highest median titres of IgA antibodies were seen in the Coeliac disease control group (544 units, range 34-27500) and the lowest in the normal control group (53 units, range 24-933). When compared to normal controls, patients suffering from CD , UC and Coeliac disease all had significantly higher titres of antibodies ($p=0.013$). However, there was no significant difference between IgA antibody titres in normal controls and affected subjects ($p=0.097$). Similarly, there was no significant difference between antibody titres in

affected subjects and patients with CD ($p=0.082$), UC ($p=0.213$) or Coeliac disease ($p=0.142$). No significant differences were noted between CD and UC ($p=0.591$), CD and Coeliac disease ($p=0.734$) or UC and Coeliac disease ($p=0.796$).

In contrast, the highest median titres of specific IgG class antibodies to mycoprotein in serum were seen in affected subjects (68 units, range 0-987) and the lowest in normal controls (21 units, range 14-149). The titres of IgG antibodies, in arbitrary units, were considerably lower than those observed for IgA antibodies (Table 4.2).

Levels of IgG specific for mycoprotein were significantly elevated in affected subjects when compared to normal controls ($p=0.019$), and were marginally higher than the levels observed in disease controls (Figure 4.4). There was no significant difference in the IgG antibody titres to mycoprotein in affected subjects compared to CD ($p=0.779$), UC ($p=0.370$) or Coeliac disease ($p=0.09$). Normal controls did have significantly lower titres of IgG antibodies to mycoprotein compared to CD ($p=0.012$) and UC ($p=0.023$), but not Coeliac disease. Again, the levels of specific IgG class antibodies to mycoprotein were not significantly different between the different disease control groups.

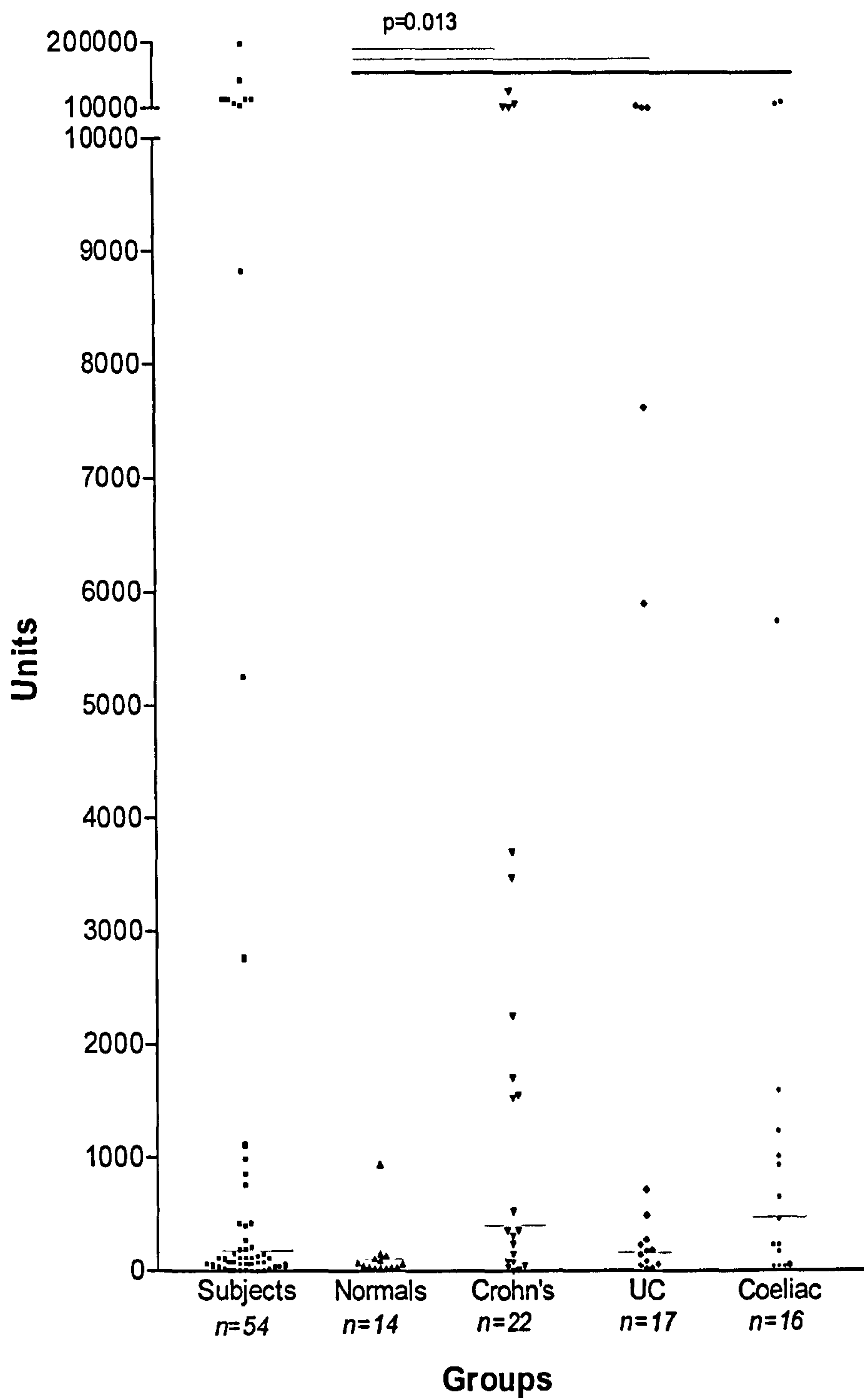


Figure 4.3 Serum IgA titres to mycoprotein

Group	anti-MP IgA antibody titre	anti-MP IgG antibody titre
	Median (Range)	Median (Range)
Subjects	115 (0-193000)	68 (0-987)
Normal	53 (24-933)	21 (14-149)
Crohn's Disease	443 (0-60700)	57 (11-544)
Ulcerative colitis	230 (26-17800)	47 (0-186)
Coeliac disease	544 (34-27500)	29 (0-535)

Table 4.2 Comparison of IgA and IgG anti-Mycoprotein Antibody Titres

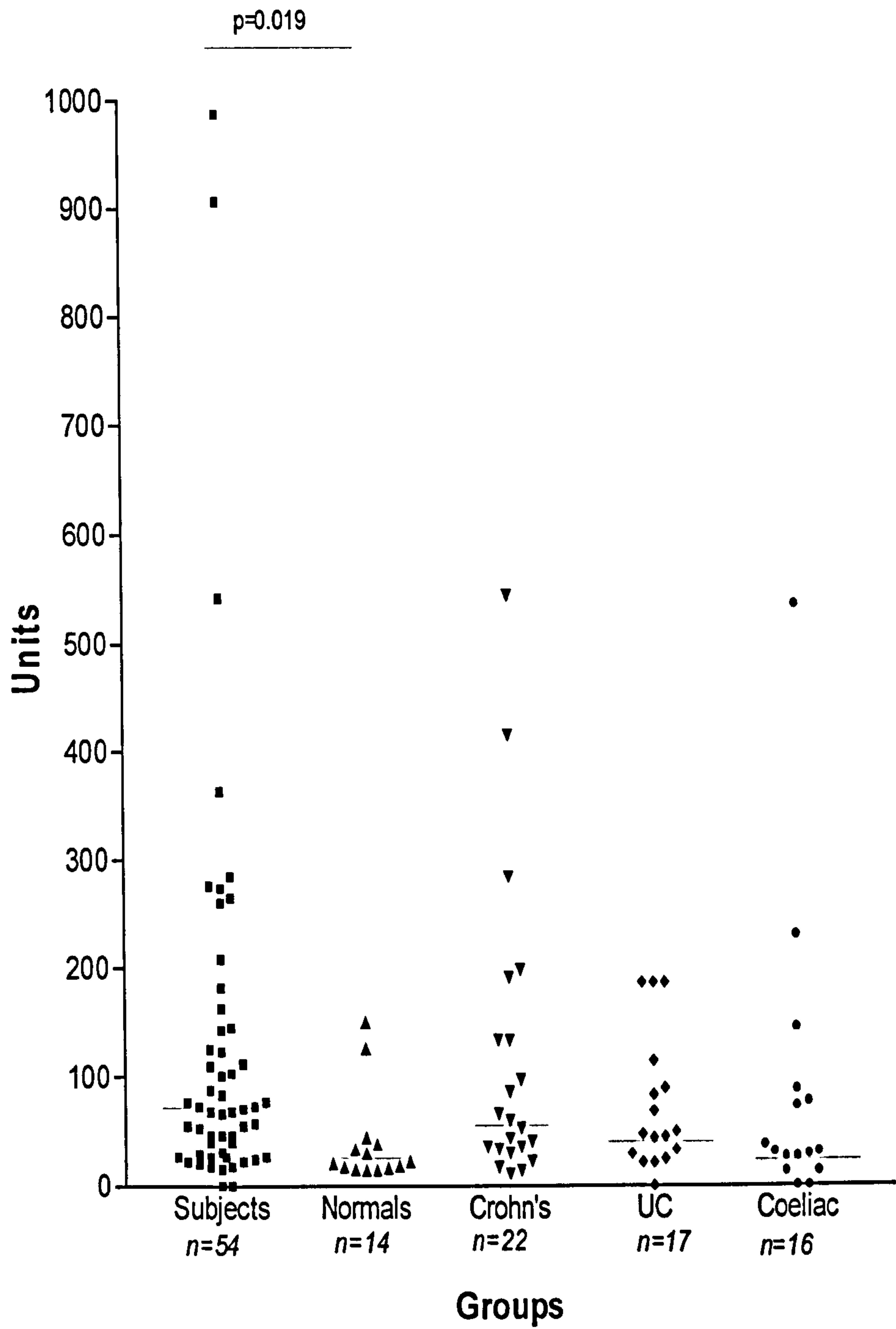


Figure 4.4. Serum IgG titres to mycoprotein

4.3.2 Serum IgA & IgG Antibodies to Egg

In the case of specific IgA class antibodies to egg protein (Figure 4.5), the highest median titres were seen in Coeliac disease control patients (184 units, range 15-24300) and the lowest in normal controls (51 units, range 16-257). Compared to normal controls, the levels of specific IgA antibodies to egg in affected subjects and Coeliac disease controls were significantly elevated ($p < 0.01$). However, titres in CD and UC controls were not significantly different from normal controls. The higher titres of IgA antibodies to egg protein, seen in affected subjects compared to CD controls, just reached a statistically significant level ($p = 0.049$), but were not significantly different when compared to UC or Coeliac controls.

Coeliac disease control patients showed the highest titres of specific IgG antibodies to egg albumin (226 units, range 0-1429) and UC controls the lowest titres (33 units, 0-939). In contrast to the levels of antibodies to mycoprotein, IgA and IgG titres to egg albumin were broadly similar (Table 4.3). However, no significant differences in IgG antibody titres to egg albumin were seen between any of the groups tested (Figure 4.6).

Scatter plots have been used (figures 4.3 to 4.6) to illustrate the wide spread of values seen in all our assays and thus the considerable overlap between these groups of individuals. A horizontal line marks the median value for each group tested.

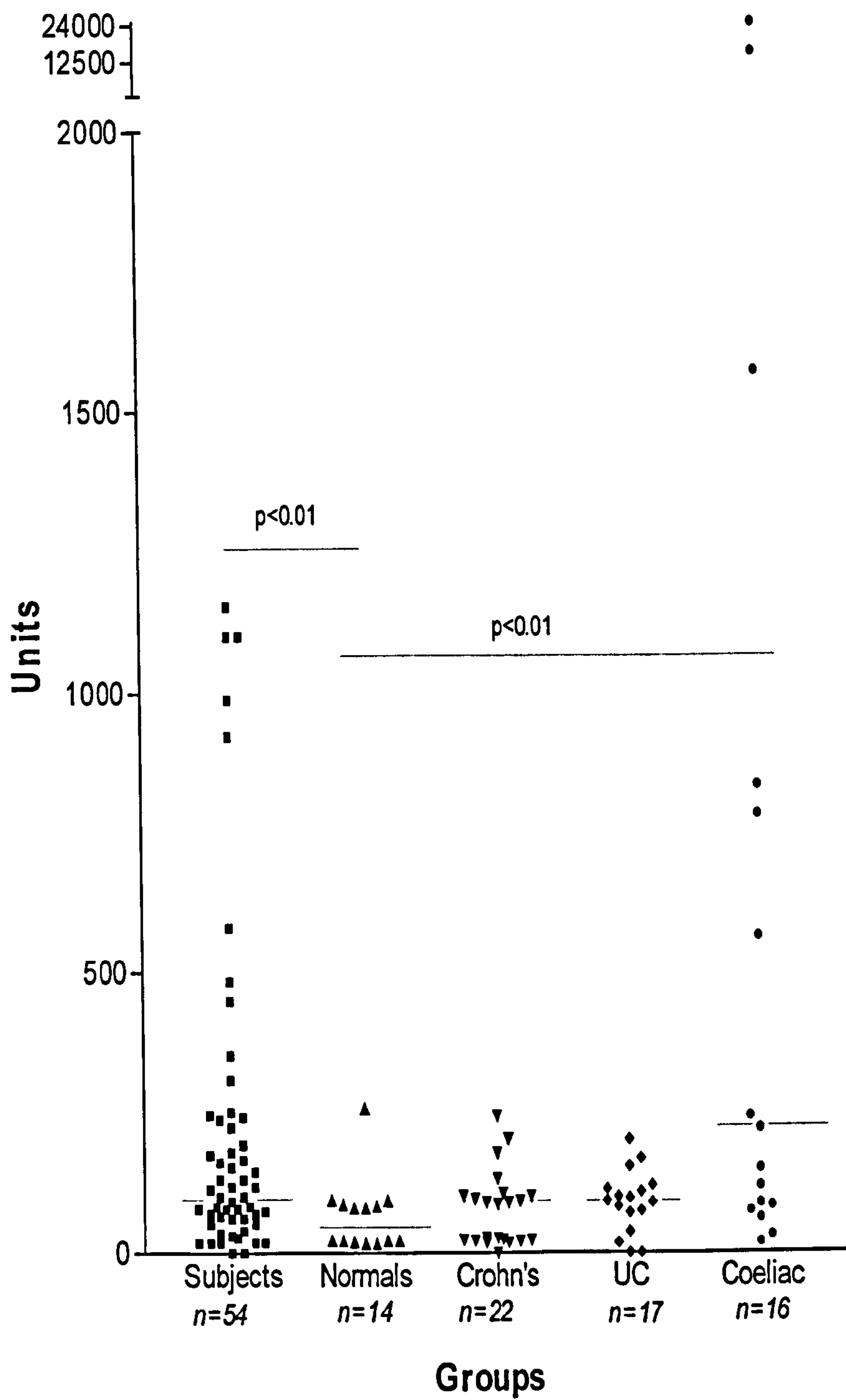


Figure 4.5. Serum IgA titres to egg albumin

Group	anti-egg IgA antibody titre	anti-egg IgG antibody titre
	Median (Range)	Median (Range)
Subjects	106 (0-1154)	117 (0-800)
Normal	51 (16-257)	165 (0-822)
Crohn's Disease	90 (0-243)	65 (0-734)
Ulcerative colitis	94 (0-202)	33 (0-939)
Coeliac disease	184 (15-24300)	226 (0-1429)

Table 4.3 Comparison between IgA and IgG titres to egg albumin

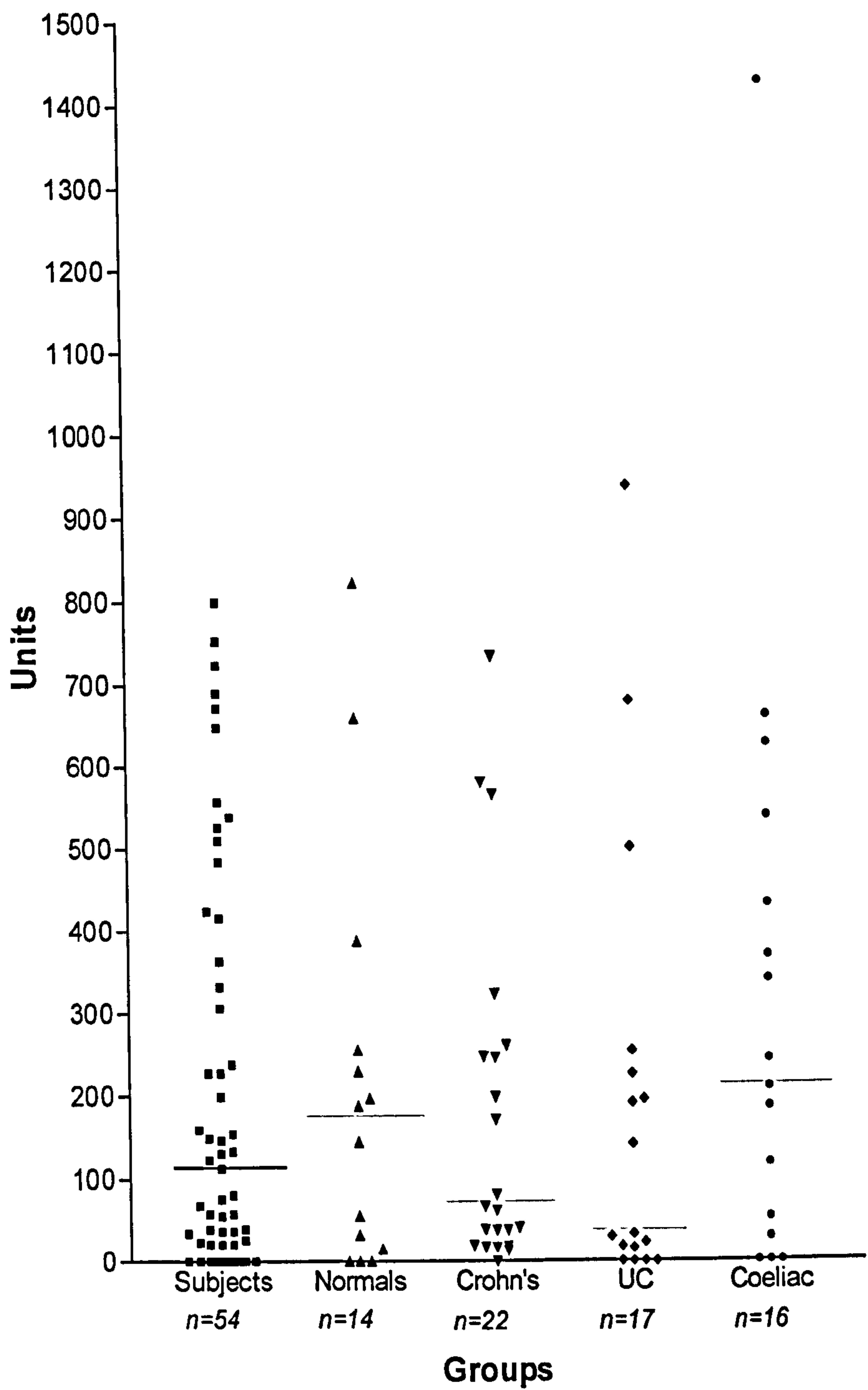


Figure 4.6 Serum IgG titres to egg albumin

4.3.3 Effect of atopy on IgA & IgG Titres in Affected Subjects

Further statistical analysis (Mann Whitney test) of the affected subjects was carried out depending on the presence ($n=22$) or absence ($n=32$) of atopic disease. There was no significant difference in the titres of IgA and IgG antibodies to either mycoprotein or egg albumin. However, there may be a trend toward a significant difference in IgG class antibodies to mycoprotein between atopic and non-atopic subjects (Table 4.4).

	Atopic	Non-atopic	<i>p</i> value*
	Median (range)	Median (range)	
IgA to mycoprotein	139 (0-193000)	101 (0-33300)	0.553 (NS)
IgG to mycoprotein	152 (0-1154)	77 (0-1101)	0.054 (NS)
IgA to egg albumin	74 (0-987)	60 (0-905)	0.529 (NS)
IgG to egg albumin	123 (0-750)	76 (0-800)	0.650 (NS)

Table 4.4 Effect of Atopy on IgA and IgG Titres in Affected Subjects

*Mann Whitney Test

NS= Not significant

4.3.4 Effect Of Disease Activity On IgA & IgG Titres In Disease Controls

The presence of active disease in IBD patients was judged by the presence of elevated levels of IgG ($>10\mu\text{g/ml}$), albumin ($>26\mu\text{g/ml}$), α -1 antitrypsin ($>19\mu\text{g/ml}$) or haemoglobin ($>5\mu\text{g/ml}$) on WGLF analysis. As previously stated (section 3.2.0), this has been shown to correlate well with clinical scores of inflammatory bowel disease activity such as the CDAI (89,261).

Table 4.5 demonstrates that in CD control patients, there was no significant difference between titres of either IgA or IgG antibodies to either mycoprotein or egg albumin, when patients with active and inactive disease were compared.

Similarly, Table 4.6 shows that in UC control patients, there was no significant difference between titres of either IgA or IgG antibodies to either mycoprotein or egg albumin when patients with active and inactive disease were compared.

	Active CD (<i>n</i> =14)	Inactive CD (<i>n</i> =8)	<i>p</i> value*
	Median (range)	Median (range)	
IgA mycoprotein	1036 (0-60700)	252 (13-3469)	0.207 (NS)
IgG mycoprotein	63 (14-544)	47 (11-133)	0.394 (NS)
IgA egg albumin	95 (19-243)	23 (0-179)	0.125 (NS)
IgG egg albumin	126 (13-734)	29 (0-260)	0.142 (NS)

Table 4.5 Effect of disease activity on IgA & IgG titres in CD controls
 * Mann Whitney test

	Active UC (<i>n</i> =11)	Inactive UC (<i>n</i> =6)	<i>p</i> value*
	Median (range)	Median (range)	
IgA mycoprotein	279 (31-11600)	123 (25-17800)	0.514 (NS)
IgG mycoprotein	49 (21-186)	31 (0-186)	0.393 (NS)
IgA egg albumin	99 (0-202)	88 (0-121)	0.421 (NS)
IgG egg albumin	33 (0-939)	110 (18-253)	0.725 (NS)

Table 4.6 Effect of disease activity on IgA & IgG titres in UC controls
 * Mann Whitney test

4.3.5 Effect of previous ingestion of mycoprotein on IgA and IgG titres in normal controls

Of the normal controls, 6 could recall having previously consumed mycoprotein and the remaining 8 were quite definite that they had never consumed the foodstuff. Comparison of IgA and IgG titres to mycoprotein and egg albumin showed no significant difference between these two groups.

4.4.0 ELISA For IgA Antibodies In Whole Gut Lavage Fluid

One subject underwent whole gut lavage as part of this study (Subject-5, female aged 18 years). IgA antibodies to mycoprotein were detectable in whole gut lavage fluid using the same method as used for serum. At a dilution of 1:2, a level of 34 arbitrary units per ml was detected

4.5.0 ELISAs For Total & Specific IgE in Serum

A wide spread of values (figure 4.7) for total serum IgE was noted in the subjects (median 35 kU/L, range 5-3589 kU/L). 21% of subjects were found to have an abnormally high level of total IgE (>114kU/L).

However, on testing for the more clinically relevant specific IgE levels (Figure 4.8) only four subjects (subjects 5, 16, 28 and 33) had any evidence of specific IgE antibody to mycoprotein at a 1 in 2 dilution i.e. a very concentrated sample. Even in these individuals, the levels detected were extremely low.

In addition, there was no correlation between levels of total IgE and specific IgA or IgG class antibodies to either mycoprotein or egg.

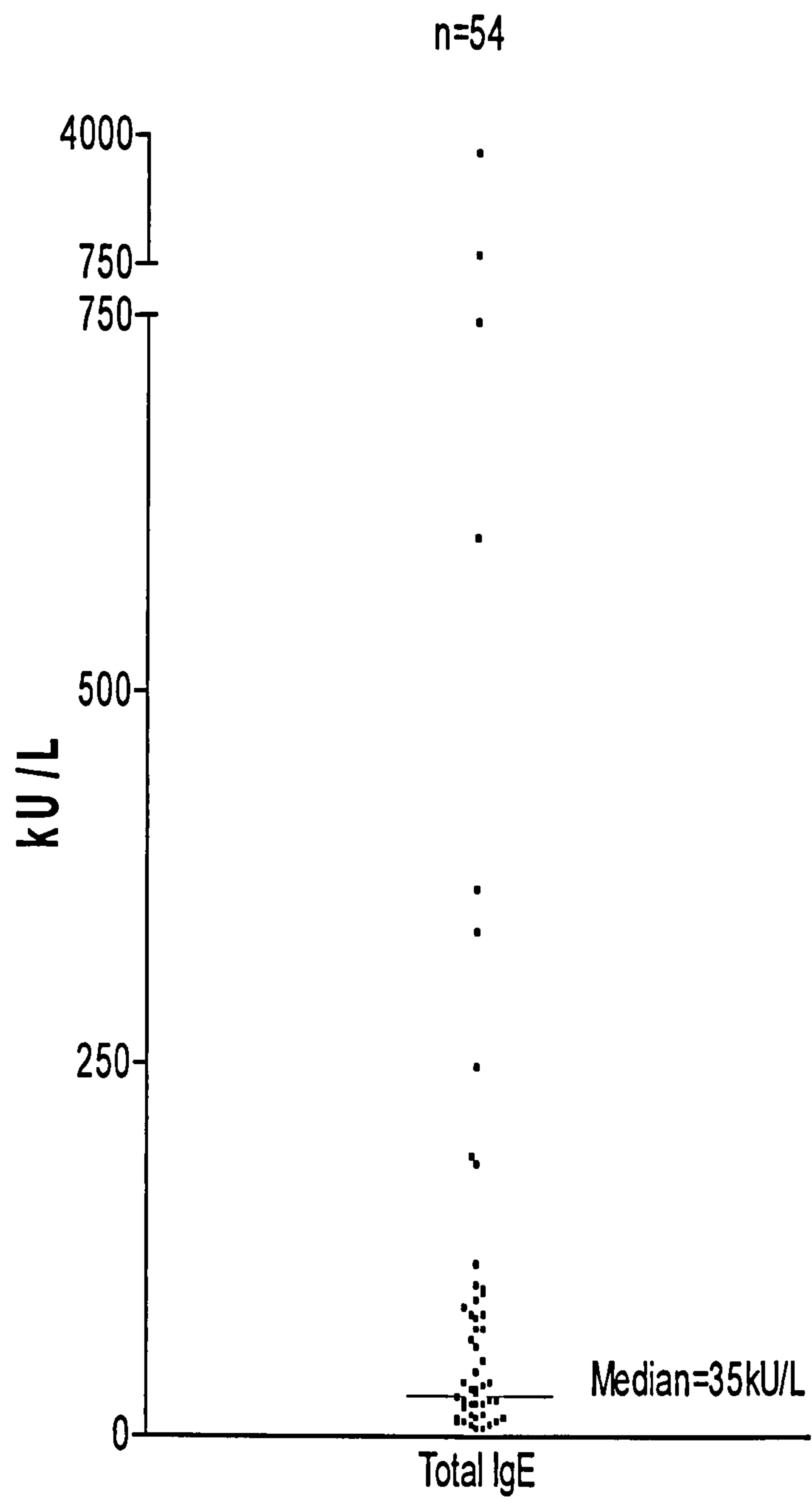


Figure 4.7 Spread of total IgE values for affected subjects

Mycoprotein-specific IgE

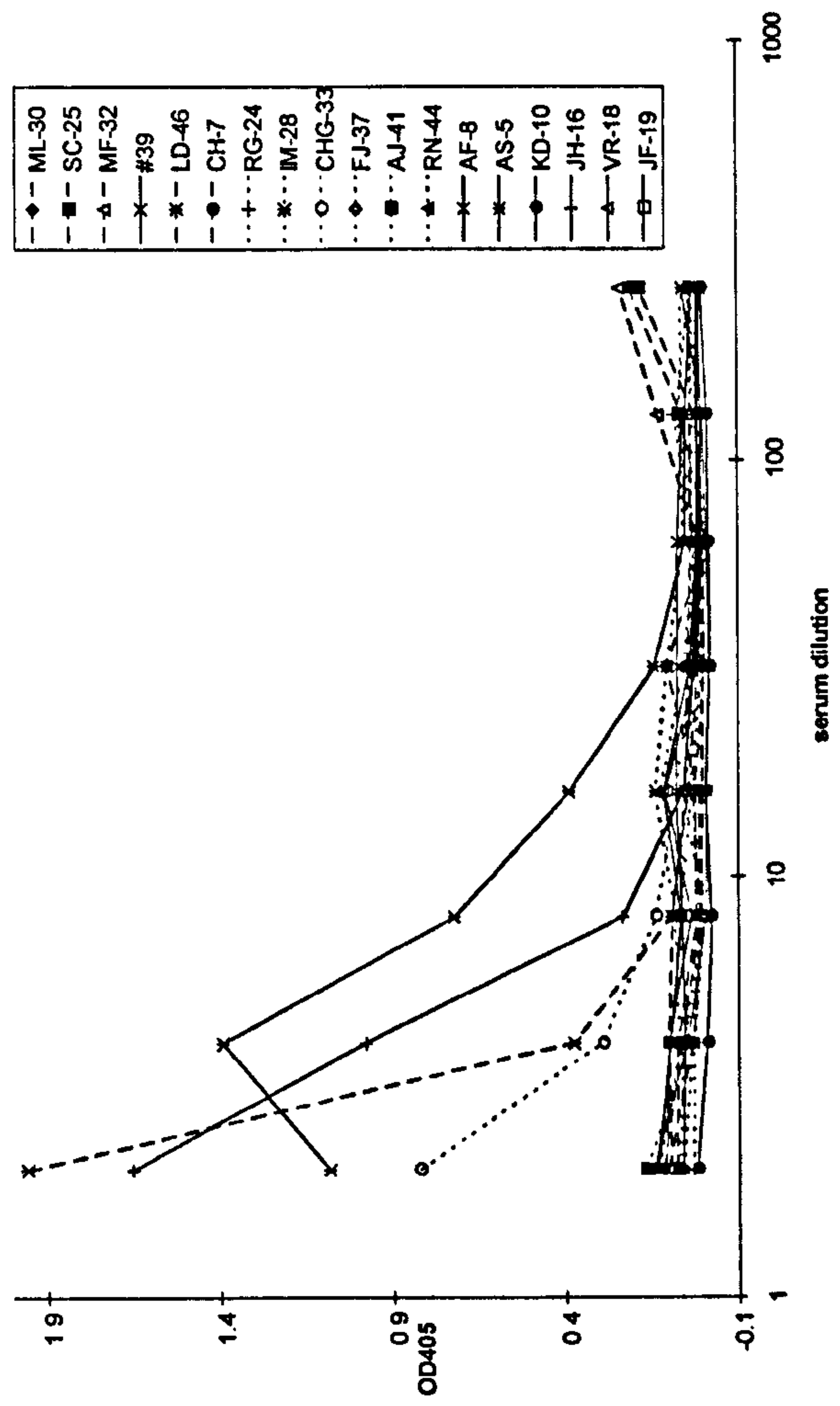


Figure 4.8 Serum IgE titres to Mycoprotein in Serum

4.6.0 ELISAs for mycotoxins

No evidence of production of specific IgA or IgG antibody to any of the mycotoxins tested (zearalenone, deoxynivalenol, or diacetyl scirpenol) was found (data not shown).

4.7.0 Competition Assays

Competition assays were performed on all those subjects and disease controls showing high levels of mycoprotein-specific IgA antibodies. No individual in the normal control group showed high levels of mycoprotein-specific IgA antibodies and so this group is not represented here.

4.7.1 Competition ELISAs with Mycotoxins, Egg & Mycoprotein

The competing antigens used were zearalenone (ZL), deoxynivalenol (DON), diacetyl scirpenol (DAS), egg protein mixture (EGG) and mycoprotein (MP). These toxins are not soluble in water. DAS and DON were serially diluted in chloroform, followed by ethanol and then ELISA diluent. DON was dissolved using this method, but DAS remained a suspension. ZL was initially dissolved in ethanol and then ELISA diluent and remained in solution. Initial experiments indicated that the solvent had little effect on the competition assay (data not shown).

Results have been expressed in bar chart form. A separate graph has been used for each different competing antigen; DAS, DON, ZL, Egg, MP. Each graph shows median percentage inhibition of IgA and IgG anti-MP antibodies for both subjects and control

groups. Error bars indicate 95% confidence intervals (calculated by the Wilcoxon rank test).

The higher a given value, the more toxin has bound to the antibody and prevented it binding to the MP attached to the plate. Conversely, a negative value indicates that the toxin has not inhibited the binding of the antibody to the MP attached to the plate, and has possibly enhanced this interaction. The subjects and disease controls were compared for the ability of the toxin to inhibit or enhance binding of the IgA or IgG antibodies to the MP bound to the plate. One subject showed enhanced binding to the antibody bound to the plate when the toxins were added in competition. This subject was not used in the subsequent analyses comparing the groups.

In the absence of competing antigen (figure 4.9) there was no difference seen in the shape of the titrated curves produced by the affected subjects and disease controls for either IgA or IgG antibodies. The optical density of the most concentrated sample of the curves in each affected subject was allowed to develop to a value of 1 OD unit.

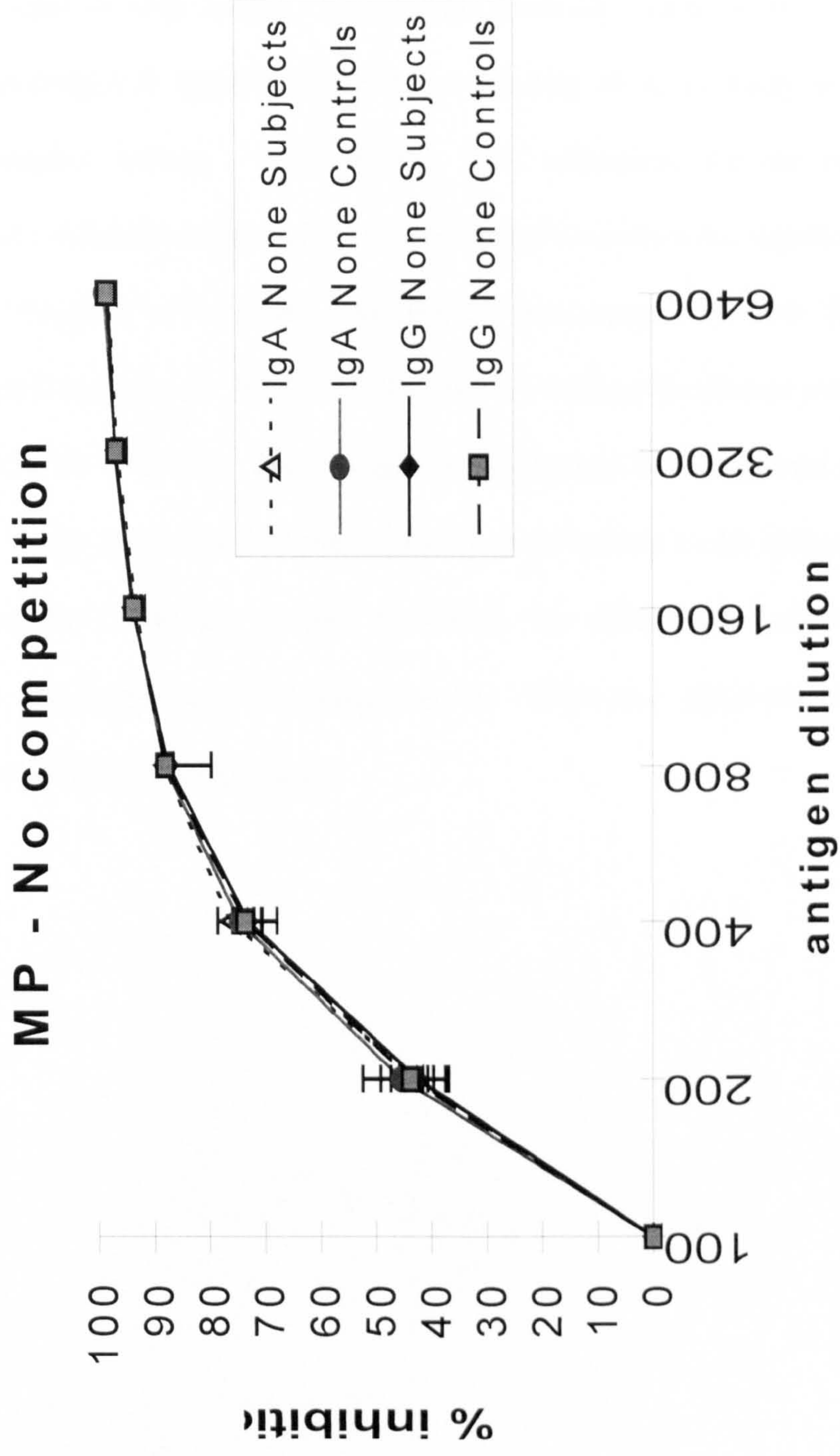


Figure 4.9 Mycoprotein alone-no competing antigen in subjects (n=16) and controls (n=14)

Diacetyl-scirpenol (DAS) produced very little inhibition of either IgA or IgG antibody binding to the plate in both subjects and disease controls (figure 4.10). The values obtained for percentage of specific inhibition of binding were, in many of the cases, found to be negative values. In competing IgA antibodies, for the two highest concentrations of DAS added, the values for the disease controls were significantly more negative. At 100µg/ml of inhibitor, the median percentage inhibition for affected subjects was 12.42% (range -13.36% - 33.15%) and the median for disease controls was -46.6% (range -120.9% - 32.4%). The competing antigen may have increased the binding of the antibody to the plate in the disease controls as compared to the subjects. As the concentration of the competing antigen decreased, the differences between the two groups became non-significant, indicating that the differential influence of the toxin decreased as its concentration decreased.

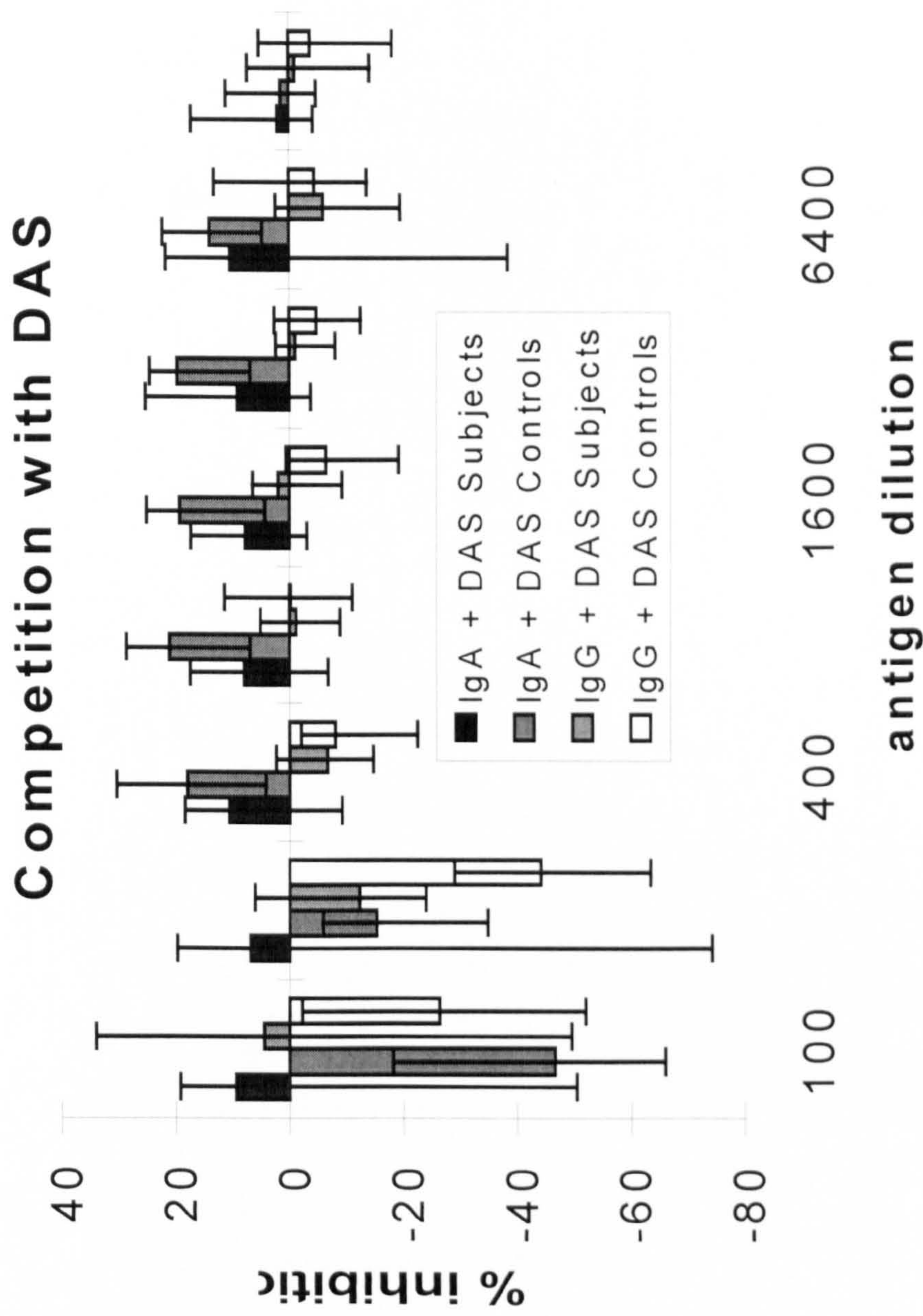


Figure 4.10 Competition ELISA- Diacetyl-scirpenol (DAS) in subjects (n=16) and controls (n=14)

There was no difference between the disease control patients and the affected subjects in the inhibition by DON (figure 4.11). Therefore, if DON has had any effect on these subjects, it is probably not through prevention or enhancement of antibody binding and is more likely to be through some other mechanism such as inhibiting gastric motility.

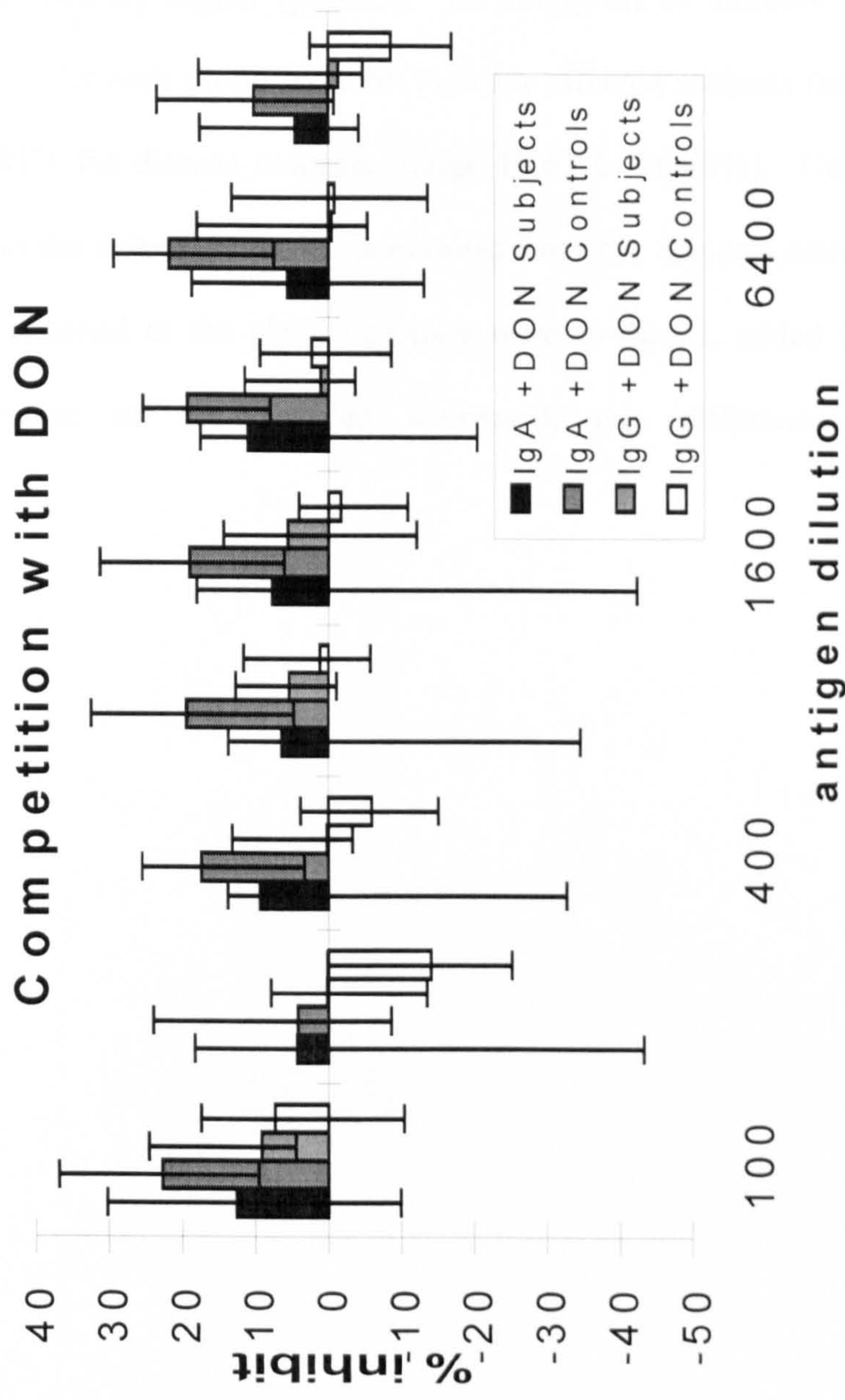


Figure 4.11 Competition ELISA- Deoxynivalenol (DON) in subjects (n=16) and controls (n=14)

Levels of percentage inhibition for zearolenone were much higher than for the other two toxins in both subjects and disease controls (figure 4.12). For IgA competing antibodies, at the highest concentration of ZL added, the values obtained for the disease controls were significantly higher ($p < 0.05$). At 100 μ g/ml of inhibitor, the median percentage inhibitions for each group were 65.71% for affected subjects (range -47.31% - 89.96%) and 75.81% for disease controls (range 10.62%- 96.83%). Compared with antibodies present in the disease controls, antibodies from the subjects were more likely to bind to the MP attached to the plate than they were to the ZL added to the samples. As the concentration of toxin added decreased, this difference became insignificant.

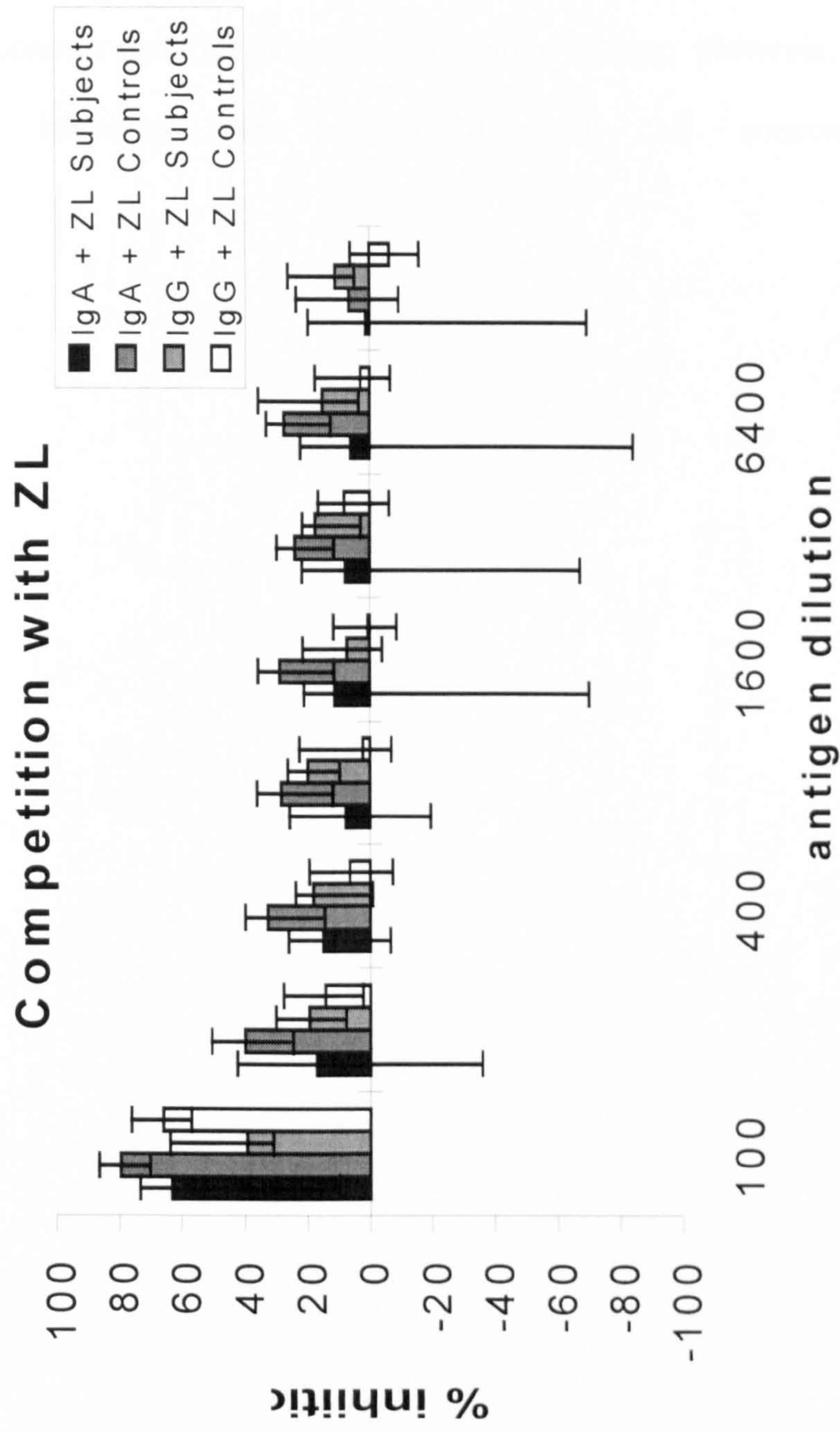


Figure 4.12 Competition ELISA- Zearalenone (ZL) in subjects (n=16) and controls (n=14)

On the whole, in subjects, the addition of egg to the serum had very little effect on the binding of the antibodies to the MP attached to the plate (figure 4.13). A few of the disease controls had higher levels of inhibition by egg. However, for both groups, the median inhibition was about 10% at all concentrations of egg.

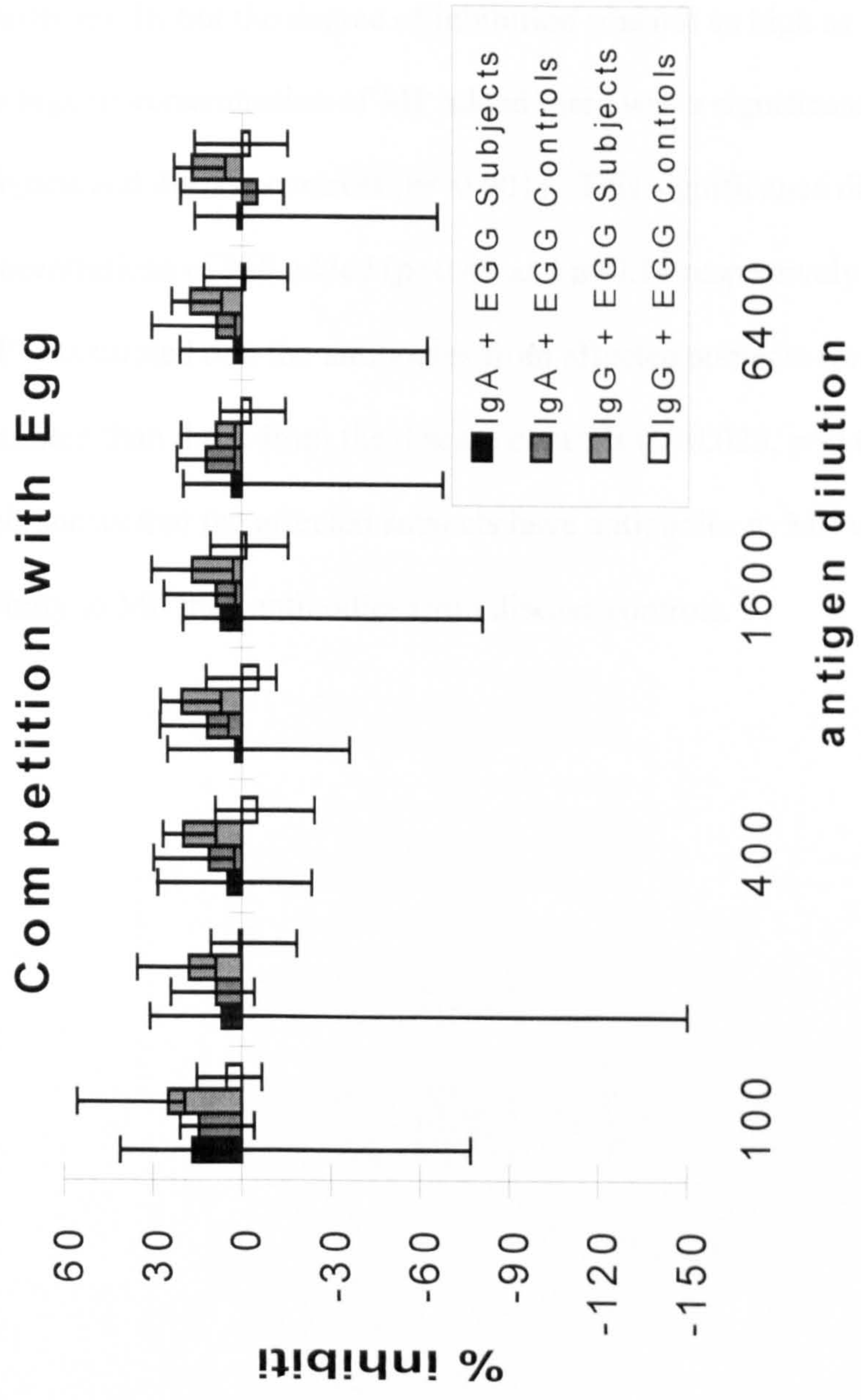


Figure 4.13 Competition ELISA- Egg in subjects (n=16) and controls (n=14)

The addition of mycoprotein as a "self-inhibitor" gave more interesting results. In the affected subjects, the addition of MP to the sample almost totally inhibited any binding of the antibody to the immobilised MP on the plate (figure 4.14). The disease controls gave similar results but the degree of inhibition was not as high as in the affected subjects. At the highest concentration of MP added there was a significant difference between the subjects and disease controls ($p < 0.001$). This significance disappeared for the next two concentrations of MP added ($p=0.49$ and $p=0.14$ respectively.) However as the level of MP was titrated out, the antibodies from affected subjects were significantly more inhibited than those from the disease controls ($p=0.025$, $p=0.002$, $p=0.004$, respectively). This shows that the affected subjects have antibodies to MP which have a much higher affinity to MP than antibodies from disease controls.

Competition with MP

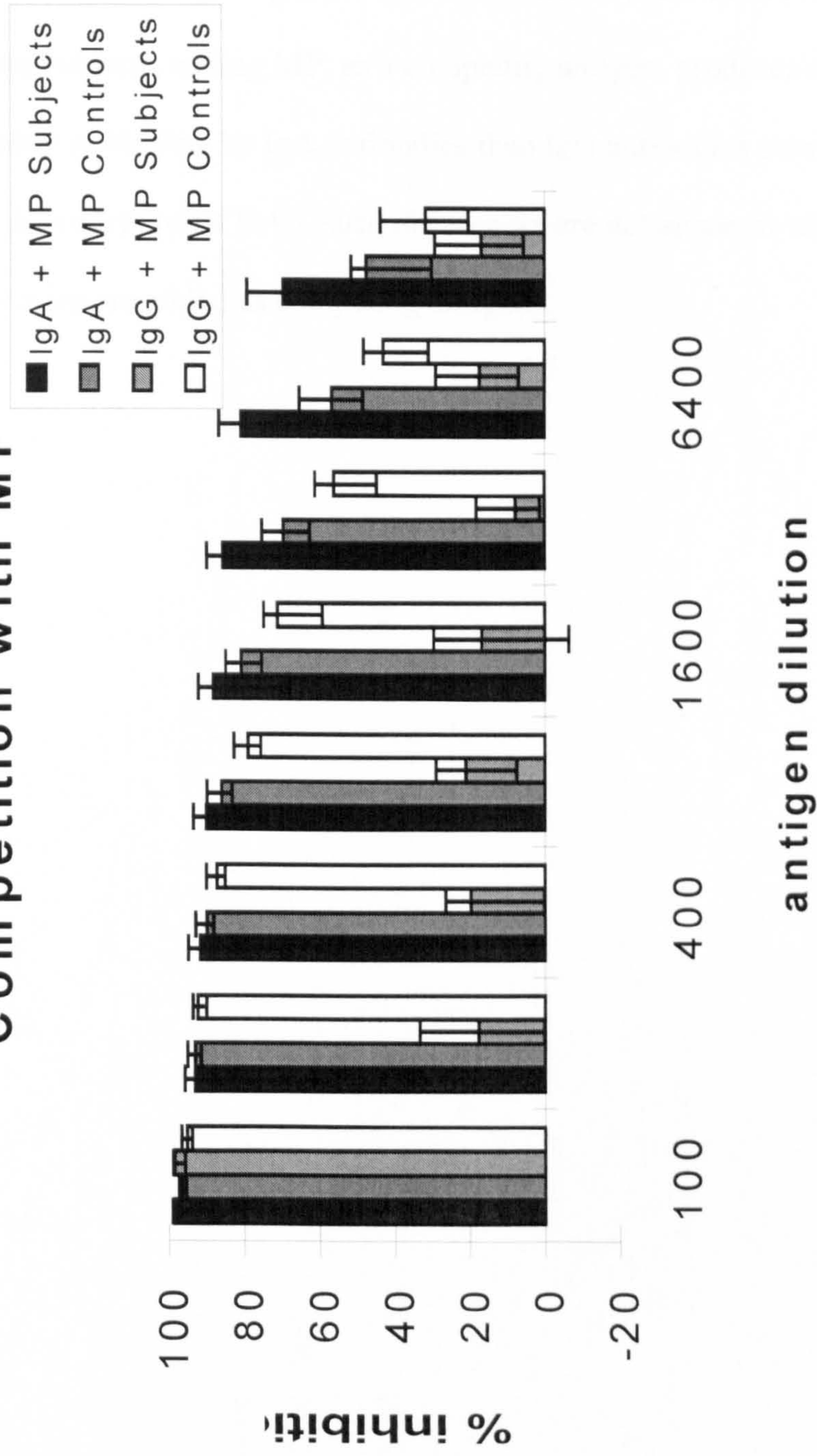


Figure 4.14 Competition ELISA- Mycoprotein (MP) in subjects (n=16) and controls (n=14)

Table 4.7 refers to the influence of the different competing antigens on the relative inhibition of binding of IgA and IgG antibodies to plate-bound MP in affected subjects. In these subjects, adding MP, as a competing antigen, produces significantly more inhibition of binding by IgA antibodies than IgG antibodies across a range of dilutions. With the exception of DAS, such differences are not apparent when the various mycotoxins are added as competing antigens.

Competing Antigen	Antigen Dilution						
	100	200	400	800	1600	3200	6400 12800
DAS	0.599	0.431	0.568	0.049	0.066	0.026	0.057 0.393
DON	1.00	0.646	0.555	0.768	0.511	0.511	0.431 0.646
ZL	0.165	1.00	0.694	0.189	0.948	0.325	0.264 0.149
EGG	0.115	0.264	0.470	0.237	0.115	0.212	0.101 0.066
MP	0.555	0.0006	0.0001	0.0001	0.0001	0.0001	0.001 0.001

Table 4.7 Inhibition of IgA Binding Compared with Inhibition of IgG Binding by Different Competing Antigens in Subjects (n=16)

Table 4.8, similarly, looks at these effects in disease controls. Some toxins, particularly DON cause significantly more inhibition of binding of IgA antibodies than IgG antibodies in these controls.

Competing Antigen	Antigen Dilution					
	100	200	400	800	1600	3200
DAS	0.407	0.025	0.001	0.023	0.004	0.001
DON	0.047	0.042	0.005	0.047	0.010	0.025
ZL	0.047	0.011	0.020	0.147	0.010	0.056
EGG	0.300	0.159	0.028	0.042	0.068	0.038
MP	0.171	0.184	0.089	0.004	0.007	0.002

Table 4.8 Inhibition of IgA Binding Compared With Inhibition of IgG Binding By Different Competing Antigens
in Controls (n=14)

When we look specifically at the effects of the various mycotoxins on inhibition of IgA binding, in subjects compared with disease controls (table 4.9) we can see that there is little difference. However, adding MP itself as a competing antigen causes significantly more inhibition of IgA binding in the subject group compared with controls.

Competing Antigen	Antigen Dilution					
	100	200	400	800	1600	3200
DAS	0.013	0.120	0.161	0.195	0.568	0.500
DON	0.299	0.678	0.276	0.276	0.350	0.161
ZL	0.020	0.132	0.077	0.062	0.195	0.078
EGG	0.836	0.917	0.604	0.213	0.406	0.276
MP	0.010	0.795	0.324	0.087	0.011	0.002

**Table 4.9 Inhibition of IgA Binding By Different Competing Antigens -Comparison Between
Subjects (n=16) and Controls (n=14)**

Finally, table 4.10 shows that the various mycotoxins do not differ significantly in their ability to inhibit binding of IgG in subjects compared with controls. There are, however, significant differences seen when egg or mycoprotein is added as the competing antigen.

Competing Antigen	Antigen Dilution					
	100	200	400	800	1600	3200
DAS	0.022	0.006	0.568	0.795	0.195	0.678
DON	0.378	0.120	0.108	0.436	0.350	0.836
ZL	0.050	0.775	0.551	0.364	0.337	0.223
EGG	0.004	0.015	0.020	0.043	0.029	0.033
MP	0.008	0.00001	0.00001	0.00001	0.0002	0.0001
					0.002	0.062

Table 4.10 Inhibition of IgG Binding By Different Competing Antigens-Comparison Between Subjects (n=16) and Controls (n=14)

4.7.2 Competition ELISA with *S. cerevisiae*

In subjects and controls, the addition of *S. cerevisiae* as a competing antigen has no significant effect on the binding of either IgA or IgG antibodies.

4.8.0 Endoscopic & Histological Appearance of Gastric Mucosa Pre & Post Mycoprotein Challenge

4.8.1 Clinical Features

Three subjects, one male, two female, age range 34-66 years, underwent paired upper gastrointestinal endoscopies using intravenous sedation with midazolam. The dose of midazolam was titrated according to subjects' conscious level and ranged between 4mg and 6mg per procedure (mean 5mg, SD 1mg).

No subject experienced vomiting post mycoprotein challenge but all three complained of bloating and nausea. These symptoms settled spontaneously in two subjects but the third (subject 35, the oldest subject to undergo invasive testing) required treatment with intramuscular metoclopramide 10mg. This relieved the feeling of nausea within 15 minutes.

4.8.2 Macroscopic Endoscopic Features

All three subjects tested had evidence of some upper GI pathology- two had a hiatus hernia with associated oesophagitis, and one had a moderately severe, *Helicobacter pylori* associated duodenitis. However, only one subject, with oesophagitis, had ever

complained of any upper gastrointestinal symptoms (in this case, symptoms of acid reflux). Post challenge, one of three subjects showed subjective endoscopic macroscopic evidence of an acute gastritis, but this was not confirmed objectively on microscopic examination. There was no evidence of a “wheal and flare” reaction described by other authors using endoscopic challenge methods (114,115).

4.8.3 Microscopic Features

There was no evidence of acute gastritis following oral challenge with the above dose of mycoprotein in any of the three subjects examined. One subject had a *Helicobacter pylori* associated antral gastritis which was present prior to exposure to mycoprotein. There was no evidence of an excess of lymphocytes or eosinophils in any of the gastric biopsies either pre or post challenge with mycoprotein.

Endoscopy (subject 35)	Pre-challenge	Post-challenge
Oesophagus	Hiatus hernia with oesophagitis.	Hiatus hernia with oesophagitis.
Stomach	3 tiny (<3mm) flat polyps in body of stomach. No gastritis or ulceration.	Flat polyps as before. No gastritis or ulceration.
Duodenum	Normal. No evidence of duodenitis or ulceration.	Normal. No evidence of duodenitis or ulceration.

Endoscopy (subject 14)	Pre-challenge	Post-challenge
Oesophagus	Hiatus hernia with oesophagitis.	Hiatus hernia with oesophagitis.
Stomach	Normal. No evidence of gastritis or ulceration.	Linear erythema radiating from pylorus. Mild antral gastritis.
Duodenum	Normal. No evidence of duodenitis or ulceration.	Normal. No evidence of duodenitis or ulceration.

Endoscopy (subject 47)	Pre-challenge	Post-challenge
Oesophagus	Normal. No abnormality seen.	Normal. No abnormality seen.
Stomach	Normal. No evidence of gastritis or ulceration.	Normal. No evidence of gastritis or ulceration.
Duodenum	Erosive duodenitis.	Erosive duodenitis.

**Table 4.11 Macroscopic features of gastric mucosa, pre & post
mycoprotein challenge**

Subject 35	Pre-challenge	Post-challenge
Antrum	Gastric mucosa within normal limits. No H. pylori.	Gastric mucosa of antral type with no evidence of histological gastritis.
Body	Gastric mucosa within normal limits. No H. pylori.	Normal body mucosa only.
Subject 14	Pre-challenge	Post-challenge
Antrum	Gastric antral type mucosa. Only a sparse chronic inflammatory cell infiltrate in lamina propria. No H. pylori. Normal.	Gastric antral type mucosa. Sparse chronic inflammatory cell infiltrate in lamina propria with 1 cluster of lymphocytes. No evidence of active inflammation, H. pylori or intestinal metaplasia.
Body	Body type mucosa with some loss of surface epithelium. Focal chronic inflammatory cell infiltrate in lamina propria. No active inflammation or H. pylori.	Body type mucosa. Mild infiltrate of chronic inflammatory cells in lamina propria. Eosinophils are not a feature. No H. pylori.
Subject 47	Pre-challenge	Post-challenge
Antrum	Gastric antral type mucosa with mixed inflammatory cell infiltrate in lamina propria. Polymorphs seen in gland epithelium. Prominent lymphoid aggregates. Moderate numbers of H. pylori. H. pylori associated gastritis.	Gastric antral type mucosa with mixed inflammatory cell infiltrate in lamina propria. Polymorphs seen in gland epithelium. Eosinophils are sparse. Prominent lymphoid aggregates. Moderate numbers of H. pylori. H. pylori associated gastritis.
Body	Body type mucosa. Mild mixed inflammatory cell infiltrate with occasional polymorphs in glands. Small numbers of H. pylori seen. No evidence of intestinal metaplasia.	Body type mucosa. Mild mixed inflammatory cell infiltrate. Polymorphs are not seen. No H. pylori seen. No evidence of intestinal metaplasia.

Table 4.12 Microscopic features of gastric mucosa, pre & post mycoprotein challenge

4.9.0 Isotope Gastric Emptying Studies Pre & Post Oral Mycoprotein Challenge

Only one subject (subject 27) male, aged 42 underwent paired isotope gastric emptying studies. Table 4.13 shows the t-half values (time taken for half the ingested test meal to leave the stomach) in minutes for both the affected subject (subject 27) and a normal control (male, aged 30). The times given are corrected values generated by the Elgems (General Electric) software program from the radioisotope decay curve.

In the baseline, pre-challenge study, the affected subject has a shorter T-half time than the normal control. Both values, however, lie within a previously calculated normal range. The T-half times were also longer in both the affected subject and the normal control following an oral challenge with 5g of cooked mycoprotein, but again, both these values are still within the normal range. In these two individuals, at least, the consumption of an oral challenge consisting of 5g of cooked mycoprotein did lead to some prolongation of solid phase gastric emptying.

However, even this prolongation did not exceed the previously calculated normal range and in view of the extremely small numbers involved, no real conclusion can be drawn from this observation.

	T-half (minutes)	
	Pre-challenge	Post-challenge
Subject 27	49.09	57.34
Normal control	67.89	77.99

Table 4.13 Isotope Gastric Emptying Studies

Chapter 5- Discussion

In this thesis, I have described a group of subjects who all experienced some form of physical or clinical reaction following the ingestion of the novel, mycoprotein based foodstuff, Quorn[®]. Particular attention has been paid to those individuals reporting symptoms of nausea, bloating and vomiting following consumption of this foodstuff, who make up the majority of cases. As the descriptions of the reactions suffered are strikingly consistent, it has been assumed that each one has been the victim of the same phenomenon.

5.1.0 Summary Of Findings

Affected subjects differed significantly from controls in that they were found to have higher titres of IgG antibodies to mycoprotein ($p=0.019$) and IgA antibodies to egg ($p=0.019$) than normal controls. They also had higher titres of IgA antibodies to egg ($p=0.049$) than Crohn's disease controls. No significant difference in titres of IgA antibodies to mycoprotein were seen between affected subjects and normal controls. However, the range of values for IgA antibodies to mycoprotein in all groups tested varied widely, particularly in affected subjects (0-193000 units). It is possible that the absence of any difference between affected subjects and normal controls could represent a Type II error, or a failure to recognise a true difference. All three disease control groups had higher titres of IgA antibodies to mycoprotein when compared to normal controls ($p=0.003$) but only Crohn's disease ($p=0.012$) and Ulcerative colitis ($p=0.023$) controls had significantly higher titres of anti-mycoprotein IgG. With the exception of Coeliac

disease controls showing higher titres of IgA antibodies to egg than Crohn's disease controls ($p=0.012$), there were no significant differences in any antibody class to either mycoprotein or egg in any of the disease control groups.

The presence of atopic disease in affected subjects did not significantly influence antibody titres. Only four subjects had detectable levels of mycoprotein-specific IgE antibodies, and even then, only in very low concentrations. In Crohn's disease and Ulcerative colitis patients, disease activity as measured by elevated levels of IgG, albumin, α -1 antitrypsin or haemoglobin in WGLF did not significantly affect antibody titres to either mycoprotein or egg. However, Table 4.5 does illustrate a trend towards higher titres of all antibodies tested in patients with active Crohn's disease. This does not reach statistical significance, but the small numbers of patients involved (14 active, 8 inactive) may again lead to the possibility of a Type II error. No such differences were seen in Ulcerative colitis patients. Affected subjects showed no evidence of antibody production to any of the three mycotoxins tested. Competition ELISAs revealed no evidence of any cross reaction between mycoprotein and egg, or between mycoprotein and *S. cerevisiae*.

There was no evidence of any irritant effect on the gastric mucosa in affected subjects undergoing upper gastrointestinal endoscopy after ingestion of a mycoprotein challenge. There was, however, a high prevalence of minor upper gastrointestinal pathology in affected subjects at baseline (i.e. pre-challenge) endoscopy. Ingestion of mycoprotein did lead to a slight prolongation of solid-phase gastric emptying as measured by a well

validated radioisotope method, but this did not exceed values seen within a previously defined normal range. These findings will now be discussed in more detail.

5.2.0 Potential Sources of Bias Within Study Population

From the outset, it must be stressed that this group was self-selected, a fact which must be borne in mind in terms of potential bias within the study. All had identified the possible link between their own reaction and the ingested mycoprotein, and had gone to the trouble of reporting their experiences to the manufacturer. This in itself implies a high degree of motivation and is borne out by their initial behaviour in writing to describe their experiences, and subsequently, their willingness to persevere with questionnaires, blood samples and even more invasive tests. This motivation appears to derive from purely altruistic principles and not from any hopes of seeking compensation from the manufacturer for the discomfort suffered. Indeed, the majority of letters to the manufacturer were at pains to point out that the correspondence was not intended as a complaint but rather as a means of gaining a better understanding of what had happened.

Therefore, the study population was identified on the basis of their own description of a reaction which had been experienced some time prior to our investigations. These descriptions are subject to recall bias, being based on the subjects' own recollection of events which had occurred up to 20 months previously. However, they were the only data available upon which any observation could be based.

The field of food related disease, whether in terms of allergy, over- or under-nutrition is fraught with the problems associated with recall bias. Retrospectively collected food diaries depend on an adequate and accurate recall of foods taken which may be beyond the capabilities of a significant minority of the population. Diarists may feel obliged to fill in what they feel they ought to be eating (ie what the investigator wants them to eat) rather than what they actually consumed. Of course, as previously discussed in the methodology section, all medical interactions between doctors and their patients depend on the acquisition of information from the patient. The information obtained, whether through an interview or the taking of a medical history, or through a questionnaire, will always be subject to recall bias. It is an important point and one which has to be borne in mind when interpreting any patient derived information.

Some specific points have been raised in the case of information derived from questionnaires. In particular, any information divulged in response to a questionnaire relies on patient recall of an event or a diagnosis. The ability to accurately recall events will depend on the patient's cognitive function as well as on factors which are specific to that patient. For example, an event or diagnosis may be recalled because of its association with something which seems quite trivial to the doctor but is of special importance to the patient. It is important to ensure that the patient's understanding of that event or diagnosis is correct and matches with what the investigator's understanding of what has been described.

The true scale of the problem, and therefore the denominator in terms of actual number of cases, is very difficult to assess. As stated in the introduction to this thesis, the number of reports reaching the manufacturer has remained constant over the past few years at around 200 cases per annum (Dr G. Rodger, Marlow Foods Ltd, personal communication, data on company file). This, of course, may only represent a small fraction of those suffering a reaction. Not everyone suffering an adverse event will associate it with the ingestion of mycoprotein, nor will they necessarily write to alert the manufacturer. However, in the case of food borne infections and food poisoning, it has been suggested that there may be up to nine silent, unreported cases for each reported case. This very rough estimate suggests that there may be up to 2000 cases of adverse reactions due to mycoprotein in the United Kingdom per annum. In 1998, the year this study began, 93,932 cases of “food poisoning” were reported in England and Wales alone (<http://www.phls.co.uk/> Official website of the Public Health Laboratory Services, England and Wales). It can be seen that, numerically speaking at least, reactions to mycoprotein foodstuffs are a very small problem.

Marlow Foods Ltd. had conducted an internal investigation of the serial and batch numbers of each product implicated in a reaction wherever this information was available. They concluded that there was no evidence of any particular batch of manufactured mycoprotein implicated in these reactions, indicating that it was unlikely that contamination of the product had occurred prior to leaving the factory (Dr G. Rodger, Marlow Foods Ltd, personal communication).

5.3.0 Demographic Considerations

There was no evidence of geographical clustering of cases (with the previously mentioned exception of two cases in Derby, but both bought in different supermarkets in different months of the same year). The majority of those affected, in this sample at least, were female, but the male to female ratio was not strikingly different. There may be a number of explanations for this finding. The majority of affected subjects were attracted by the healthy image of mycoprotein products, a non-animal protein source low in fat and high in fibre, an image which may be more attractive to health conscious females. Female subjects may also be more willing to report a reaction, and more likely to take the time to sit down and write to the manufacturer than males. Previous studies have also shown a similar pattern in that replies from females outnumber those from males (175,176). Thus, it is unlikely this result is a true predisposition to adverse reactions to mycoprotein amongst female subjects.

5.4.0 Potential Role of Atopic Disease and IgE-mediated Mechanisms

Affected subjects reported a high prevalence (48%) of atopic conditions, such as asthma, eczema, allergic rhinitis or hayfever. Of the 90 subjects who replied to the questionnaire, a history of atopic disease was commonest in those who had experienced the non-vomiting reactions (64% reported atopic history). This rose to 100% in the two subjects experiencing anaphylactic type reactions and was 66% in those who developed a skin rash. The lowest rate of atopic disease (44%) was seen in the subjects who had experienced the vomiting reaction (see figure 4.1). This group was further sub-divided

on the basis of whether reactions occurred on the first or on subsequent exposure to mycoprotein. Of those vomiting the first time they ingested the foodstuff, 48.9% had a history of atopic disease, compared with 64.2% of those reacting on subsequent exposure (see figure 4.2). These figures are considerably higher than the figure of 27% quoted in the large population based study of food intolerance by Young *et al* (174). In that study, rates of self-reported reactions to food were higher in those individuals with a history of atopy (28%) than in those with no such history (15%). So the presence of atopy is an important factor in a substantial number of individuals who experience reactions to food. Atopic disease is cited by the American Gastroenterological Association as one of the features in the history of a reaction which increases the likelihood of a diagnosis of food allergy (151). It would appear that, in the case of individuals who reacted adversely to mycoprotein, atopic disease is less important in those subjects who experienced a vomiting reaction, compared to those who experienced skin rashes or anaphylaxis. The prevalence of atopy is lowest in those subjects who vomited the first time they ingested the foodstuff. Perhaps some of these individuals have a predisposition to allergic-type reactions not associated with atopic disease. It seems more likely, however, that the vomiting reaction has a non-allergic aetiology. The relatively lower incidence of atopy in this group may be further “soft” evidence for this.

Atopy has a strong familial component (269). An individual's lifetime risk of developing an atopic disease is increased between 20-40% if one parent is affected, rising to between 40-60%, if both parents are atopic and up to 80% if both parents express the type of

atopic disease (270). It might, therefore, have been useful to investigate whether reactive individuals shared any particular HLA-types or could be further categorised in this way.

Atopic individuals generally have high serum levels of total IgE. Indeed, the clinical features of atopic diseases are mediated by IgE-class antibodies. As the non-vomiting reactions (anaphylactic-type and skin rashes) followed the classical pattern one might expect in IgE mediated reactions, it is not surprising to find a higher incidence of atopy in this group.

IgE mediated reactions are known to fall into two categories depending on the length of time between exposure to allergen and onset of reaction. The more typical immediate-phase reaction is mediated by degranulation of previously sensitised mast cells with release of vasoactive inflammatory mediators, such as histamine. Histamine release causes smooth muscle contraction and increased permeability of capillaries and arterioles with “wheal and flare” skin lesions. Antigen-induced degranulation of intestinal mast cells has been shown to stimulate motor and secretory function in the small and large intestine of the rat (271-274). This immediate phase IgE response is important in a variety of atopic conditions including allergic rhinitis and atopic dermatitis.

However, mast cell degranulation also releases other chemokines and cytokines, such as eosinophil and neutrophil chemotactic factors, granulocyte/macrophage colony stimulating factor (GM-CSF), IL-3 and IL-5 which lead to the recruitment of other inflammatory cell types to the site of inflammation (260). The attraction and activation of neutrophils, macrophages and monocytes is likely to result in a delayed, but more

prolonged inflammatory response. Such a mechanism has been suggested to underlie many food hypersensitivities (10). The time scale for the development of a delayed phase IgE response is in the order of 6-8 hours, persisting for 24 hours and this would fit very well with the clinical history described by those subjects experiencing the vomiting reaction after mycoprotein ingestion.

In the affected subjects in this study, the atopic group did have higher median levels of total IgE than the non-atopic group, as would be expected. However, only four subjects out of 54 (subjects 5, 16, 28 and 33) had measurable levels of mycoprotein specific IgE class antibodies and these were only detectable in high concentrations of serum (1:2 dilution). One of these subjects (subject 28) gave no previous history of atopic disease, which is an unexpected finding. Whether this is indeed the case, and subject 28 is truly not atopic, or whether this person had some difficulty in understanding the questionnaire is impossible to say with any degree of certainty. The fact that he demonstrated a high titre of mycoprotein- specific IgE antibody is very suggestive of an atopic tendency. The mean total IgE level in serum for the whole group of affected individuals was slightly elevated (mean 187, normal range <114kU/L). However, in view of the presence of two or three very high "outlier" values within this group, which elevated the mean value, a more realistic reflection might be the median value, which at 35 kU/L, was well within the normal range. We did not employ RAST testing, another method to assay food-specific IgE class antibodies, but relied instead on the ELISA technique. Different results may have been obtained if RAST testing had been used as it is a very sensitive technique, but lacks specificity, producing significant numbers of false positive results.

In conclusion, from the results presented, it is unlikely that the vomiting reaction is truly mediated by IgE, or IgE dependent mechanisms. However, the absence of such IgE-mediated mechanisms does not exclude the possibility of some other, humorally-mediated immunological effect.

5.5.0 Role of IgA & IgG Antibodies In Serum & Whole Gut Lavage Fluid

The presence of detectable levels of food specific antibodies in the sera of patients with inflammatory bowel disease and Coeliac disease has traditionally been ascribed to the phenomenon of increased intestinal permeability (81,275). Increased permeability has been shown to be a feature of Coeliac Disease and to a lesser extent, Crohn's disease and Ulcerative colitis (276,277). The diseased or inflamed intestine may present a less effective barrier to the passage of substances from the lumen across the intestinal wall. This means that the underlying immune cells in the deeper layers of the gut wall are exposed to a greater antigenic load in the form of partially digested food components. The increased antigen presentation leads to activation of T-helper cells, enhanced proliferation of lamina propria B-cells which differentiate into immunoglobulin secreting plasma cells and increased antibody production. Antibodies of both IgA and IgG classes, to a variety of food antigens, have been demonstrated in patients with untreated Coeliac disease (278,279). In a study of 14 untreated Coeliac patients, Hvatum *et al* (275), showed elevated levels of IgA antibodies to the milk and egg proteins, α -lactalbumin and ovalbumin, and elevated IgG subclass antibodies, particularly IgG4 subclass, to β -

lactalbumin, α -lactalbumin and ovalbumin. This may be part of a normal, non-pathological humoral immune response to dietary antigen (280). However, IgG4 is the IgG subclass most commonly implicated in allergic reactions to dust, pollens and bee venom (281) and thus, may also be implicated in allergic reactions to foods. Unfortunately, we did not examine IgG subclass responses, but it would be unusual to see an allergic reaction mediated by IgG4 with no corresponding activation of IgE.

As previously stated, normal subjects will produce IgG antibodies to a range of food components. These may be seen in 9.2% of randomly tested blood donors (282) and are more common in the presence of atopic diseases, such as eczema. These antibodies are a physiological phenomenon reflecting exposure of the individual to these foods. One would expect, however, that over time the levels of such antibodies would fall as, through repeated exposure over time, the body becomes “tolerant” to that particular immunological stimulus. It may be of some significance then, that titres of IgA- and IgG class antibodies to both mycoprotein and egg were measurable at all in the sera of affected subjects.

IgA is the predominant immunoglobulin class secreted by mucosal surfaces including the intestinal epithelium. Although in health, very little is detectable in the serum, increased levels are suggestive of overproduction at the mucosal level, with a possible “overspill” into the systemic circulation. Of the 54 affected subjects, seven had no detectable titre of IgA class antibodies to mycoprotein. Of this group of seven, two also had no IgA class antibody titres to egg protein. There is a possibility that this may simply be a chance

finding, of little consequence, but IgA deficiency is associated with Coeliac disease and may be present in up to 20% of such individuals (2). It is possible that these two individuals (subjects 36 and 38), may in fact be hitherto undiagnosed Coeliac patients. Furthermore, IgA deficiency removes one of the mucosal barrier's most effective lines of defence against enteric infection and may predispose to such infections, an alternative explanation for their symptoms. Unfortunately, serum immunoglobulin levels were not tested in this group, so no definite diagnosis can be made.

In the case of antibodies to mycoprotein, it was demonstrated that all groups of disease controls had significantly higher titres of IgA class antibodies than normal controls. There was no significant difference in titres between normal controls and affected subjects. In the case of IgG antibodies, however, the situation is different. Affected subjects had significantly elevated titres of mycoprotein-specific IgG compared to normal controls ($p=0.002$) and these levels exceeded those seen in disease controls. This could be taken to show that there is some breakdown of the normal immunoregulatory mechanisms, such as oral tolerance, in the affected subjects or an enhanced level of humoral immune response in these individuals compared to normal controls.

No data on intestinal permeability were available on any of the affected subjects or normal controls, and only on a few of the Coeliac disease controls. It was therefore, not possible to see if any correlation existed between the level of antibody response generated and the degree of intestinal permeability. This would have been an interesting experiment, but it is likely that no such relationship exists. There was no evidence that

the disease controls with active disease, in whom one might expect an increased intestinal permeability, had significantly higher titres of mycoprotein or egg specific antibodies, although, as discussed in section 5.1.0 there was a trend towards higher values in those with active disease.

Increased intestinal permeability has been described in a subgroup of first degree relatives of patients with Crohn's disease (283,284), and also in one study, in unaffected spouses (285) who have no evidence of clinical disease. Therefore, while increased permeability may reflect active disease in those with inflammatory bowel disease, it may also be seen in the absence of inflammation. The increased permeability seen in unaffected relatives may actually be a result of a specific genetically determined defect of intestinal permeability, associated with inflammatory bowel disease. The precise relationship between increased intestinal permeability and the presence of circulating antibodies to foods is unclear, but may be less straightforward than simply increasing the exposure of mucosal immune cells to luminal contents.

One might expect, if the titre of food specific antibodies depends purely on the degree of intestinal permeability, that the relative patterns of antibody response to mycoprotein and egg would be broadly similar. This was not the case. The pattern of IgA and IgG antibody class response to egg albumin is rather different to that seen to mycoprotein. No patient had any recent history of gastroenteritis, alcohol abuse or use of non-steroidal anti-inflammatory drugs, all of which may affect intestinal permeability.

All subjects recruited to the present study were enrolled throughout 1997. Serum samples were collected and stored to await assay over a four week period in May 1998. Data regarding the precise date on which subjects experienced their first (and subsequent) reactions to mycoprotein are incomplete. Where dates are available the information given is, as previously discussed, subject to recall bias and may not be accurate. The length of time taken to realise mycoprotein may be implicated, write to the manufacturer, become involved in the study and provide a sample of serum is variable. Of those subjects where such data is available, the shortest time taken from reaction to serum sampling was four months, and the longest 20 months. The earliest documented reaction suffered amongst affected subjects was in October 1996.

There is not sufficient data from the group of affected subjects to make any meaningful comment on whether the length of time between experiencing an adverse reaction and submitting a serum sample influences the level of antibodies detected in serum. Data from individuals suffering from enteric infections such as *Salmonella enteritidis* have shown that serum antibodies to the pathogen are absent at one year following the initial infection, while antibodies in intestinal secretions may persist as a “footprint” of infection long after this. It may not be relevant, however, to compare events occurring after exposure to an intestinal pathogen to those related to ingestion of foodstuffs, where one might expect a greater degree of immune tolerance and down-regulation. The mucosal immune response exists to neutralise the former while tolerating the presence of the latter. The one subject who did undergo WGL, showed high levels of antibodies in her serum and did indeed have evidence of anti-mycoprotein antibodies in the WGLF. She

was a particularly reliable historian who had experienced a reaction just over 12 months previously. Again, as repeated time and time again in this thesis, her case alone does not prove that WGLF antibodies persist for many months in all individuals but it does at least tell us that antibodies can be detected even 12 months after a reaction has occurred.

It should be noted, however, that there was considerable overlap in the range of antibody titres obtained in all groups to both foods tested. With this in mind, the results of the serum levels of IgA and IgG antibodies must be viewed with caution. Care should be taken to avoid reading too much into these results. In addition, Quorn[®], although, still a novel foodstuff, is fairly universally available in supermarkets in the United Kingdom. Therefore, it is possible that some of the control subjects had experienced reactions to the foodstuff, invalidating their use as a control. Of the five normal controls who had consumed mycoprotein, none had experienced any problems or adverse reactions. The disease controls were never asked specifically whether they had personally experienced any problems after consuming mycoprotein products, but none had ever reported any reaction which was in any way similar to that described by the subjects.

It would have been of great interest to perform WGL on all 54 volunteers who provided samples of serum for analysis, and to have assayed these intestinal secretions for the same IgA, IgG and IgE antibodies to mycoprotein and egg. Without the benefit of such studies we can only speculate on events at a mucosal level in the affected subjects reacting to mycoprotein. It has been a message which this laboratory has preached for many years that serum studies do not accurately reflect events at a mucosal level.

However, it would have been impractical to have obtained WGLF from each of these subjects, and while many would willingly consent to giving a blood sample, far fewer would have agreed to undergoing WGL even if it had been practical to perform these procedures at home. The original decision to perform initial screening studies in serum was made with full knowledge of the likely problems which might be encountered trying to obtain WGLF from each questionnaire respondent.

5.6.0 Competition ELISAs- Potential Role of Cross-Reactivity

The results of the competition ELISAs were interesting, as these were designed to investigate the possibility of either exposure to mycotoxins, or to some other substance which might cross-react immunologically with these toxins. Tee *et al* had previously demonstrated multiple common allergenic determinants between the fungal species *Aspergillus fumigatus*, *Cladosporium herbarum* and *F. graminearum* (12), raising the possibility that individuals allergic to these moulds could react adversely to inhaled or ingested mycoprotein. However, in common with this thesis, their results were largely negative, indicating that this theoretical risk was unlikely to be a major problem clinically.

Although levels of serum IgA in subjects and disease controls were broadly similar, the competition assays showed that there were qualitative differences between the antibodies of individuals from each of these groups. Where a difference was seen, the subjects had antibodies which had a consistently higher affinity for the mycoprotein than for either the egg albumin or mycotoxins. The significance of this is not clear, but may reflect an

immunological mechanism which would make these subjects react more violently to mycoprotein. This could have occurred by prior exposure to cross-reacting antigens. Possible mechanisms of environmental exposure might include inhalation of airborne mould spores or ingestion of foods made from cereals contaminated by moulds. Contamination of cereal crops around the world with *Fusaria* species, including *Fusarium graminearum*, and their toxins has been well documented (286). Minute quantities of mycotoxins have even been discovered in beer in Czechoslovakia (287). In the Czech study, the authors concluded that even assuming an annual beer intake of 150 litres per person, the cumulative dose of deoxynivalenol was not hazardous. It may however, be sufficient to cause priming of the intestinal immune response, awaiting the subsequent ingestion of a mycoprotein meal.

The disease controls may also have been previously exposed as they too had shown antibody responses to the mycoprotein. If the increased affinity for antigen seen in the subjects is a true result then this would indicate that exposure to mycoprotein in these subjects had indeed caused some subclinical sensitisation to the food product. This may imply some immunoregulatory defect or breakdown of oral tolerance. The fact that the majority of subjects (>80%) described a reaction to mycoprotein on their first exposure unfortunately does not bear this out, but would still be consistent with the theory of prior cross-sensitisation. Further studies would be required to see if different epitopes of the mycoprotein are recognised in the different patient groups.

In the competition assays, the most significant differences seen are in the inhibition of binding of IgA antibodies rather than IgG antibodies to mycoprotein on a solid surface compared with free mycoprotein in solution. These show that IgA antibodies have a greater affinity for bound mycoprotein than IgG antibodies. This observation may indicate that the reactions seen are mediated more at a mucosal (ie IgA secreting) level than at a systemic (ie IgG secreting) level.

One of the initial hypotheses was that these reactions may be due to sensitivity to egg albumin used in the manufacturing process of these foods. The IgA competition assays using egg as the competing antigen showed very little inhibition of binding, (<10%), at all concentrations of egg used. This indicates that egg albumin sensitivity is unlikely to be a significant factor in these reactions.

All the above observations are based on measurement of antibody levels within serum. As such, we are relying on the generation of an antibody response to a soluble or at least partially soluble antigen. Therefore, the conclusions reached based upon these results may not hold true if the clinical reaction is actually mediated by some insoluble component of the mycoprotein material which was discarded at the beginning of the experiments, during processing of the mycoprotein antigen for the ELISA process.

Serum measurements may not accurately reflect what is happening at the level of the intestinal mucosal lining, but serum has been used as it is the most convenient fluid to sample. Studying immune responses in the form of measuring levels of antibodies and

cytokines produced in the gut at a mucosal level is fraught with difficulties, not the least of which is gaining access to the intestinal tract.

One very useful technique, which does allow the researcher an insight into immune processes at the mucosal level, is whole gut lavage (WGL). This was initially described by Gaspari *et al* (83) but taken up and greatly developed by the late Professor Ferguson in Edinburgh. It has been widely used within the Gastrointestinal Unit, Western General Hospital, Edinburgh to give information on gut inflammation, blood and protein loss. One affected subject (subject 5) did undergo WGL and was found to have detectable levels of mycoprotein antigen-specific IgA antibodies in WGLF. This person also had high levels of mycoprotein-specific IgA and IgG antibodies in serum. This finding indicates that even many months after the initial exposure to antigen, and the intervening period when no antigen was ingested, some individuals are still producing detectable amounts of specific antibodies at a mucosal level.

Overall, the presence of these antibodies, whether in serum or in whole gut lavage fluid, although of interest, may represent an epiphenomenon, that is, a coincidental finding which may not have a direct causal link with the underlying aetiology of the reaction. Their presence does serve as a “footprint”, showing prior exposure to the mycoprotein, and an indication of some level of activation of the mucosal immune system. Subtle differences noted between results for IgA and IgG classes of antibodies, particularly in the competition assays, suggest that this reaction does occur more at a mucosal level than

a systemic level. Whether the presence of elevated titres of mycoprotein- specific antibodies represents a cause or an effect of the adverse reactions is unclear.

5.7.0 Investigation Of A Direct Irritant Effect On Gastric Mucosa

On the basis of the histories given by those suffering a vomiting reaction, namely, symptoms of nausea and abdominal bloating within a few hours of ingestion of the mycoprotein, followed by complete relief of these symptoms by vomiting, the possibility of a direct irritant effect on the mucosa of the upper gastrointestinal tract needed to be actively excluded. This is especially so as the majority of subjects had symptoms on the first exposure to the product with no history of prior sensitisation. The more directly invasive tests were used to investigate this possibility further. Endoscopy allows direct visualisation of the gastrointestinal mucosa and identification of the cardinal signs of inflammation if these are present. In the diagnosis of food allergy, it may be used as an adjunct to taking a good history and tests to detect food-specific IgE mediated reactions. Its main role was originally as a means of obtaining biopsy material from the gastrointestinal tract for histological examination. The histological features of biopsies from 53 children suffering from a food allergy, affecting the rectum or foregut, were reported in 1986, in a retrospective series by Goldman and Proujansky (166). The commonest presentations of allergic gastroenteritis were vomiting, abdominal pain and weight loss, affecting children aged between six months and 17 years. There was a differential distribution of microscopic abnormalities within the stomach, with the gastric antrum most frequently involved (22/22 cases) and the gastric body less frequently

(11/21). A diffuse and intense infiltration of eosinophils was noted in the antrum and a much less marked, focal infiltration in the body of the stomach.

Direct application of potential allergens to the gastrointestinal mucosa via an endoscopic catheter was reported in Germany in 1988 (114). Using the technique of intragastral provocation under endoscopic control (IPEC), they studied 30 well- characterised patients with proven food allergy and 10 previously healthy volunteers, by endoscopic administration of homogenised food suspensions. After challenge, the macroscopic appearance of the gastric mucosa changed, with the appearance of mucosal swelling and discrete area of haemorrhage. Histologically, there was evidence of increased numbers of lymphocytes in the gastric mucosa of food-allergic patients prior to challenge, but no change in the number of these cells following challenge. However, microscopic erosions of the gastric mucosa were seen following challenge.

Bischoff *et al* described a positive “wheal and flare” reaction to at least one of three colonoscopically injected food antigens within minutes of injection in 54/70 patients tested. The presence of such a mucosal reaction correlated with immunohistochemical markers of eosinophil and mast cell activation but not with serum concentrations of total or food-specific IgE (116). These invasive methods are not frequently employed in the routine diagnosis of food allergy. However, a modification of Reimann and Lewin’s technique was used to endoscopically assess the effect of an orally administered mycoprotein challenge in affected subjects. The previously noted macroscopic and

histological abnormalities (114,116,166) were particularly useful when assessing the gastric biopsy material pre and post mycoprotein challenge.

The results of the endoscopic studies were of interest. However, the nature of this project, particularly the need for subjects to travel to Edinburgh, meant that the eventual number of subjects studied was small. Therefore, in spite of each subject acting as their own internal control, it is difficult to reach meaningful conclusions from only three cases. However, some interesting observations can be made. All three subjects had some evidence of pre-existing minor upper gastrointestinal pathology although, of these, only one was symptomatic (section 4.8.1). All three subjects experienced symptoms following ingestion of the test meal but it should be stressed that the presence of symptoms alone is very subjective, and impossible to interpret as the challenge was neither placebo-controlled nor blinded. Endoscopic appearances of gastritis, with congestion of the gastric mucosa, often correlate poorly with histological features, and great care has to be exercised in the interpretation of an endoscopic finding of gastritis (288). The endoscopic and histological diagnoses were at variance in two cases and as a result, the presence of an acute inflammatory infiltrate histologically has been taken as the standard for the diagnosis of gastritis. Apart from one patient with *Helicobacter pylori* associated gastritis, histologically there was no abnormality noted in the epithelium of the gastric antrum or body prior to mycoprotein challenge. In particular there was no evidence of an excess of lymphocytes or eosinophils, either pre or post oral mycoprotein challenge.

As far as possible, efforts were made to reproduce the conditions under which the original reactions had occurred without exposing any of the volunteer subjects to undue

distress. It could be argued that the dose of mycoprotein administered, or the time for which that dose was in contact with the gastric mucosa prior to the second endoscopy was insufficient to cause a noticeable effect. Bischoff's colonoscopic reactions were said to reach their maximum at between 10 and 15 minutes post-challenge (116), so it is possible that any reaction had dissipated before the second endoscopy.

In this small number of susceptible individuals, there was a high frequency of asymptomatic, and unexpected, minor upper gastrointestinal pathology but no evidence of acute gastritis or worsening of pre-existing gastritis following oral ingestion of mycoprotein. With the *caveat* that the testing procedure may not have picked up the macro- or microscopic features of an acute reaction as above, I infer from these findings that there is no direct irritant effect of mycoprotein on the gastric mucosa in these individuals. It must be assumed therefore that the idiosyncratic vomiting reaction is due to some other mechanism, perhaps delayed gastric emptying. This may be further exacerbated by pre-existing, asymptomatic upper gastrointestinal pathology.

The finding of minor degrees of upper gastrointestinal pathology on endoscopy has been mirrored in a paediatric study from Finland which set out to determine whether children with recurrent bouts of abdominal pain included an excess of individuals suffering from food allergy. Eighty four children under the age of 15 years underwent skin prick and patch testing as well as upper gastrointestinal endoscopy and biopsy. On the basis of open elimination-challenge testing with various foodstuffs the authors found that 28/84 (33%) had evidence of food allergy and that 38/84 (45%) had evidence of minor

endoscopic pathology, with oesophagitis, lymphonodular and erosive duodenitis being the most common abnormalities (289,290).

In our study, only two subjects gave any prior history of gastrointestinal symptoms, and certainly no history of gastrointestinal disease. However, it is possible that some of these people may suffer from a latent or occult gastrointestinal disease such as Coeliac disease, although their symptomatology is not entirely suggestive of this. The possibility of undiagnosed Coeliac disease is further strengthened by the two subjects discussed in section 5.4.0 with no detectable IgA antibodies to mycoprotein or egg protein, raising the suspicion of IgA deficiency. It would have been possible to test the sera of these individuals for IgA class anti-Endomysium antibodies which are highly specific and sensitive for Coeliac Disease (2). However, as we did not have consent for this test from any of our subjects and as a positive result could have serious long-term implications in terms of treatment and lifestyle, it was decided not to proceed with this line of enquiry.

5.8.0 Effect of Mycoprotein Challenge on Solid-phase Gastric Emptying

One particular mycotoxin, deoxynivalenol, otherwise known as “vomitoxin”, has been shown to cause vomiting in rats by delaying gastric emptying via an effect on peripheral serotonin-3 receptors (291). The possibility that this may also happen in humans was explored by studying gastric emptying. Unfortunately, only one subject attended for isotope gastric emptying studies. It was particularly difficult to recruit subjects for this investigation, as it involved two whole days away from work and family. In addition,

because of the nature of the investigation and the small amount of radio-activity involved, only male subjects (already in the minority) or female subjects who were not of child-bearing age (who were reluctant to travel) could be included. In an internal, non-blinded comparison, when compared to the previously defined normal range, there was no underlying abnormality of solid-phase gastric emptying before ingestion of a mycoprotein challenge. Following ingestion, there was a slight increase in the time taken for half the test meal to empty from the stomach but even then, solid-phase gastric emptying was still well within the normal range. The results were remarkably similar to those seen in an age and sex matched normal control. Ideally, further studies of gastric emptying pre and post mycoprotein challenge need to be performed in affected subjects to address this issue properly.

5.9.0 Other Possible Factors

There was no suggestion of any excess of psychological morbidity in any of the affected subjects who returned a completed questionnaire. However, the primary purpose of the questionnaire was not to elicit a history of any such problems. The accuracy of the histories obtained by questionnaire relied entirely upon the goodwill of the subjects concerned to notify (without prompting) any history of depression, anxiety or other psychological problems. There is still considerable stigma attached to psychological or psychiatric disease, and of course, many individuals will not wish to raise the subject if it can be avoided. However, the reactions described were all temporally associated with ingestion of mycoprotein and there is really no reason to suspect that the vomiting reaction is in any way psychogenic. Similarly, the clinical features consistently described

by each subject do not fit with the rare cyclical vomiting syndrome, most commonly described in children, but known to affect adults (292).

Another possible insight into the aetiology of this reaction comes from a study performed to investigate the effects of a high fibre meal on subsequent short-term eating behaviour. In a within patient design, Burley *et al* fed 18 lean, healthy subjects a lunch containing either 11g of fibre (mycoprotein containing meal) or 3g of fibre (an isocaloric, chicken containing meal). In all other respects but the fibre content, these meals were identical. No differences were seen in eating behaviour during the meals; amounts eaten, rates of consumption and overall motivation to continue eating were identical. However, there was an 18% reduction in the amount of energy consumed at a subsequent evening meal when subjects had eaten the high fibre mycoprotein for lunch. This difference was highly significant ($p < 0.001$) indicating that the mycoprotein meal had a significant impact on subjects' late satiety, as measured by energy consumption 4-6 hours later. For the purposes of their study, the authors concluded that their data had clear implications for the control of appetite and body weight (293). However, it is of note that these otherwise healthy individuals had subtle but measurable decreases in satiety around six hours after ingestion of a mycoprotein containing meal. This made them feel sufficiently "full", six hours later, to eat an average of 18% fewer calories than they would have done following a meal containing chicken. It is possible that the bloating, nausea and eventual vomiting suffered by the affected subjects described in this thesis may be an exaggerated form of this process, which may indeed be mediated by alterations in upper gastrointestinal motility, in a spectrum ranging from the very subtle to the clinically relevant.

5.10.0 Outcome of present study

This was in effect a negative study, a matter of disappointment for the investigators, but more importantly, of reassurance to both the manufacturer and ultimately to the consumer. The present study did not identify any group of individuals at particularly high risk of adverse reactions to mycoprotein, in terms of their age, gender, past medical and allergic history and atopic status. No areas of potential concern within the manufacturing process of the mycoprotein-based foodstuffs were identified. In particular, there was no immunological evidence of any of the affected subjects having been exposed to potentially harmful mycotoxins. This is of great reassurance and does indicate that the present systems to detect the presence of such mycotoxins in any batch of mycoprotein are efficient at preventing the exposure of the consumer to these dangerous substances. As such, this study had relatively little impact on the manufacturer's policies in terms of changing labelling or manufacturing processes.

One area highlighted as obviously deficient was in the collection of an adequate dataset of information on those individuals who had experienced adverse reactions. In future, as a consequence of this study, all reactions will be recorded using the questionnaire detailed in Appendix 1.

5.11.0 Future work

The negative findings of this study are of course disappointing and yet there are a number of areas which merit further study.

The problems with recruiting subjects willing to travel to Edinburgh to undergo further testing, whether in the form of WGL, endoscopy or gastric emptying studies proved to be much greater than originally anticipated. However, given the media and lay public's increasing suspicion of the medical and scientific community in the context of innumerable high-profile cases of misconduct, to have managed to persuade five people to travel across the UK and undergo "tests" is no mean achievement.

It would be wrong to assume that a second cohort of subjects would be any more amenable to travel than the present cohort and so any further work would ideally have to avoid such travelling. This would necessitate near-patient testing and testing in a form which did not inconvenience the subjects and was as non-invasive as possible. In the present study, information regarding only the systemic immune response is available in the majority of subjects (only one subject had agreed to undergo WGL). Information on responses at the mucosal level would be very useful and would indicate whether any reaction did indeed occur at that level in response to mycoprotein.

To develop new assay techniques would of course be the subject of a further thesis in itself. A novel technique might involve direct or indirect methods of examining mucosal immune responses. Direct methods might include examination of intestinal biopsy material from affected subjects, normal and disease controls, attempting to isolate lamina propria lymphocytes in tissue culture. The results of *in vitro* stimulation with mycoprotein of lymphocytes from different patient groups could be compared with

stimulation by conventional mitogens by measuring production of proinflammatory cytokines as well as cytokines important in allergic responses such as IL-5 and IL-10. Descriptive studies of cytokine release in organ culture of small intestinal biopsy material might also give insight into events at a molecular level in the intestinal mucosa. Of course, obtaining biopsy material involves an invasive procedure in the form of an endoscopy, which again would require volunteer subjects to travel to Edinburgh.

In order to look at events at a mucosal level indirectly, I would propose to assay markers of granulocyte activity, such as calprotectin, a neutrophil enzyme, and eosinophil cationic protein or eosinophil protein X in stool samples (133-135). These three enzymes are sufficiently stable in faecal samples to allow subjects to provide a sample which could be sent by courier or by first class post to the Gastrointestinal Unit for assay. This would also allow comparison with levels of all three proteins in the stools of immunologically healthy individuals as well as with those of patients with inflammatory bowel diseases from data held in the Unit. Elevated levels of eosinophil-associated proteins would be most relevant in the context of a putative food hypersensitivity reaction. Comparison with patients with inflammatory bowel disease and coeliac disease would indicate whether any elevations observed were restricted to those suffering from hypersensitivity reactions or formed part of a more generalised non-specific inflammatory response. It would be useful to see whether any relationship existed between levels of faecal calprotectin and eosinophil proteins in food allergic patients, normal controls and inflammatory bowel diseases. A positive correlation would favour a non-specific inflammatory response involving neutrophil and eosinophil activation, while a selective,

isolated rise in ECP or EPX in the food allergic group would strongly support the pivotal role of eosinophil activation in the aetiology of such reactions.

Ideally, it would be very useful to see whether an oral challenge with mycoprotein produces measurable activation of the intestinal immune system. There are ethical problems involved in re-challenging otherwise healthy individuals with an allergen that previously made them ill, and which has the potential to cause even more serious adverse effects, including anaphylaxis. If such a re-challenge were to take place, it should be in an environment in which full resuscitation facilities and personnel are available in order to minimise the risk to the subject under study. Effectively this means either a trip to Edinburgh for the subject or the investigator travelling to a hospital or clinic close to the subject. I feel that the difficulties of the former option have already been demonstrated by the present study. The problems of the latter option, where the investigator might find himself administering test food challenges in surroundings unfamiliar to him, and where he is unfamiliar to local staff are potentially extremely hazardous. This option is difficult to justify in the investigation of a condition where avoidance of the offending substance is the only treatment required to ensure that subjects remain healthy. In view of these problems, both practical and ethical, such a re-challenge study would be very difficult to perform.

If sufficient historical WGLF samples had been available within the Unit to allow paired testing of mycoprotein antibodies in both the sera and WGLF in patients with inflammatory bowel disease, this would have given valuable insights into the mucosal as

well as the systemic response to mycoprotein in these patients. These could have been compared with the historical data obtained by O'Mahony *et al* regarding the presence of systemic and mucosal antibodies to other food antigens in this group of patients. In recent years, the WGL procedure has been rarely performed and there is no ongoing collection and storage of samples. As a consequence, historical samples are now very scarce, making the study of paired samples difficult.

The perceived prevalence of food allergy and intolerance is on the increase and many otherwise healthy individuals are keen to discover what is at the root of their symptoms of nausea, bloating and diarrhoea in response to a variety of foods. A whole industry has grown up around this field and it is possible to make a living as an alternative practitioner in many of the major cities in the UK by offering consultations, a bewildering array of tests and advice on exclusion diets to people who believe they are suffering from food allergies. In spite of this, it is apparently difficult to recruit subjects for food allergy studies in the sphere of conventional medicine. Whether this represents a growing interest in alternative therapies or a growing distrust of conventional medicine is not clear. The latter possibility is however very important in the field of food intolerance, where subjects feel that neither they nor their symptoms are taken seriously or believed by conventional practitioners. In the absence of robust, reliable, non-invasive methods of diagnosing food allergy or intolerance, this unfortunate situation is likely to persist.

5.12.0 Conclusions

In the course of this work, it has not been possible to conclusively identify any definite cause of these reactions, nor any obvious predisposing factors. The antibody studies do not support the notion that the vomiting reactions suffered by affected subjects are due to a humorally mediated, immunological reaction, or true food allergy. The role of the mycotoxins known to be produced by *Fusaria* species, namely zearalenone, deoxynivalenol and deoxyscirpenol would appear to be negligible, which is as one would hope with the strict regulations which exist for their detection during the process of manufacture. The results of competition ELISAs would support this. In the small number of subjects who attended for endoscopy, there was a surprisingly high prevalence of minor upper gastrointestinal pathology noted even before administration of mycoprotein, which might suggest some latent or sub-clinical upper gastrointestinal disease. However, these observations are based on findings in only three patients and should be viewed with great caution. Mycoprotein ingestion did lead to a prolongation of solid phase gastric emptying compared to baseline in the one affected subject who underwent testing. However, a similar effect was seen in a normal control subject, and in both cases, solid phase gastric emptying remained within the normal range. It is possible that the subtle changes in satiety seen with hours of mycoprotein ingestion described by Burnley in a study of normal, healthy volunteers (293), could reach a clinically relevant level in a small number of individuals who are exquisitely sensitive to alterations in upper gastrointestinal motility. Finally, there is the unsatisfactory possibility that this may be an idiosyncratic reaction to a foodstuff without any measurable physiological basis, a true food intolerance.

Better labelling of the product, making it obvious that it is derived from a mushroom protein, should result in fewer adverse reactions in those individuals who are aware that they are sensitive to mushrooms. However, this will have little impact on the small number of people who appear to have a predisposition to what may be an adverse effect of the product on upper gastrointestinal motility. Thankfully, the scale of the clinical problem is very small, at least in numerical terms, and once the problem is identified, there is an obvious treatment in the form of strict avoidance of this mycoprotein-based foodstuff.

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Appendix 1- Postal Questionnaire

BY ASKING THE FOLLOWING SERIES OF QUESTIONS I AIM TO BUILD UP A SHORT MEDICAL HISTORY TO ALLOW ME TO OBTAIN A FULLER PICTURE OF YOUR GENERAL HEALTH, PAST AND PRESENT. NOT ALL THE QUESTION, THEREFORE, RELATE TO YOUR EXPERIENCES AFTER CONSUMING QUORN PRODUCTS. THANK YOU FOR YOUR CO-OPERATION.

NAME **Date Of Birth**

ADDRESS

TEL NO.

GP NAME

GP ADDRESS

GP TEL NO.

PRESENTING PROBLEM
(Please give a brief account of your experience after consuming Quorn)

PAST MEDICAL HISTORY
(Include any operations or chronic illnesses e.g. asthma, eczema, diabetes, high blood pressure, heart problems or breathing problems)

DRUG HISTORY
(Include any tablets or medicines prescribed by your GP and any other remedies you may use)

ALLERGY HISTORY
As far as you know, are you allergic to anything?

e.g.	
Foodstuffs	Y / N
Dusts / Pollens	Y / N
Metal / Jewellery	Y / N
Drugs / Antibiotics	Y / N

If so, please give details

Do you suffer from any of the following:

Asthma	Y / N
Eczema / Dermatitis	Y / N
Hayfever	Y / N

FAMILY HISTORY

Are you aware of any health problems or allergies in any member of your immediate family?

Father

Mother

Sisters or Brothers

Uncles or Aunts

Grandparents

SOCIAL HISTORY

Please give brief details of the following:

Do you smoke? Y / N

Do you drink alcohol? Y / N

**MANY THANKS FOR TAKING THE TIME TO ANSWER THE ABOVE QUESTIONS.
YOUR REPLIES WILL BE TREATED IN THE STRICTEST CONFIDENCE.**

**DR ALAN SHAND
RESEARCH FELLOW
GASTRO-INTESTINAL LABORATORY
WESTERN GENERAL HOSPITAL
EDINBURGH
EH4 2XU**

Appendix 2 Abstracts arising from thesis

- 1. Shand AG, Aldhous MC, Ferguson A. An unusual cause of vomiting. Oral presentation to the Caledonian Society of Gastroenterology Autumn Meeting, Kirkcaldy, November 1998. (Withdrawn at sponsor's request)**
- 2. Shand AG, Aldhous M, Ghosh S, Ferguson A. Can serum levels of IgA and IgG antibodies to a novel fungal dietary protein differentiate between Crohn's Disease and Ulcerative Colitis? Oral presentation to the Caledonian Society of Gastroenterology Spring Meeting, Aberdeen, June 1999.**
- 3. Shand AG, Aldhous MC, Ghosh S. Circulating antibodies to dietary antigens in inflammatory bowel disease-a breakdown of bystander suppression? Gut 1999, 45(suppl V) A346. Poster presentation to 7th United European Gastroenterology Week, Rome, November 1999.**

Appendix 3 Papers arising from thesis

1. **Shand AG, Aldhous MC, Ghosh S.** Investigation into adverse gastrointestinal reactions to mycoprotein. (Submitted to Clinical & Experimental Allergy)

Appendix 4 Local ethics committee submission

LOTHIAN RESEARCH ETHICS COMMITTEE

APPLICATION FORM IN RELATION TO A REQUEST FOR ETHICAL APPROVAL - 1997

THIS FORM IS VALID FOR USE ONLY UNTIL THE END OF 1997

(All information must be completed in typeface)

- 1 REGISTER ENTRY** (The information in Section 1 will be held on a computer database as the Register of Proposals and will be open to the relevant NHS bodies.)

1.1 Title of the proposed research:

**CLINICAL STUDIES OF THE MECHANISMS OF ADVERSE REACTIONS
TO MYCO-PROTEIN**

- 1.2 Aims of the study:** (A brief explanation in layman's terms of the purpose of the research and how it will advance medical knowledge or benefit individual subjects.)

Myco-Protein is related to mushrooms and to other fungi, and foods based on myco-protein are now widely consumed. The manufacturers of myco-protein foods receive, from time to time, letters from individuals who feel that they have had an adverse reaction to one or more of these foods. The aim of this study is to establish the underlying mechanism behind these reported adverse reactions, by clinical interviews with a selection of people affected and a limited number of the diagnostic tests.

- 1.3 Method:** (A simple description including a brief outline of what is required of the research subjects including whether the project design is open, random, stratified, within subject, blind, what measurements will be made, what statistical analysis will be applied and whether statistical advice has been sought on the size, power and design of the project.)

An open study, which will involve a structured clinical interview and clinical examination of up to 40 people who report that they have had adverse reactions to myco-protein in food. Further investigation on the likely cause of the reaction will be carried out in groups of 6 affected people at a time, involving upper GI endoscopy in 6 people, whole gut lavage test in 6 people, a radioisotope test of emptying of the stomach in 6 people and also a radioisotope test of the stomach emptying in 6 volunteers who have not reported any adverse reactions to myco-protein in food. Finally, tests for antibodies to the constituents of myco-protein foods will be set up and assays performed in serum and intestinal fluid samples from healthy individuals, diseases which cause intestinal inflammation or allergic reactions, and from people who have had clinical reactions to myco-protein.

- 1.4 Name and address of the organisation carrying out the research:**

Gastrointestinal Unit, Western General Hospital, Edinburgh EH4 2XU

- 1.5 Name and address of the principal researcher:** (The member of the research team to whom all correspondence will be directed.)

Professor Anne Ferguson

- 1.6 Name(s) and qualifications of the research team:** (Indicate supervisor)

Immunology Graduate and Clinical Research Fellow to be appointed.

- 1.7 Name the premises in which the research will be carried out:**
(e.g. hospital[s], doctor's surgery, etc.)

The Western General Hospital.

- 1.8 Name(s) of the relevant NHS Trust(s):**

The Western General Hospital NHS Trust.

- 1.9 Medical support and other facilities available:**

A dedicated Clinical Fellow will offer medical support to the work. Limited other facilities (eg. for endoscopy studies of gastric emptying and for Out Patient Clinic assessment) have been costed and the resources of the Trust will be used with appropriate re-imbursement made.

1.10 Can you confirm that you have complied with Scottish Office and any relevant national guidelines? (ABPI, ARSAC, Guidelines on post-marketing surveillance etc.):

YES

1.11 Is this multi-centre research?

NO

2 SUBJECTS

2.1 Indicate whether subjects will be:

OUTPATIENTS	<input checked="" type="checkbox"/>
INPATIENTS	<input type="checkbox"/>
HEALTHY VOLUNTEERS IN THE COMMUNITY*	<input checked="" type="checkbox"/>
OTHER (Please specify)	<input type="checkbox"/>

People who have written to the manufacturers of myco-protein foods, indicating that they have had an adverse reaction to such foods.

* If students, has the relevant institution's Ethics Committee been consulted? YES/NO

2.2 For Volunteer Studies, do you accept the responsibility for ascertaining that, on recruitment and during the study, volunteers do not have, or acquire, a health status contra-indicating the study? (Volunteers for non-therapeutic research need not be in perfect health providing that their participation will not affect their underlying condition. Researchers should be satisfied about the state of health of such persons and a statement of any current medication being taken should be required from all recruited.)

YES

2.3 How many subjects will be involved?

40 myco-protein sensitive subjects; 6 healthy volunteers; serum and intestinal secretions from up to 100 GI Unit patients (material already collected and stored therefore there will be no additional procedures or discomfort to these latter group).

2.4 List inclusion criteria:

Individuals who have reported one of two types of adverse reaction to myco-protein containing foods - gastrointestinal reaction, with vomiting 2 -4 hours after having a meal containing myco-protein; and the rare allergic type reactions with swellings of the lips and mouth on contact with the substance in an atopic individual.

2.5 List exclusion criteria: (Such as pregnancy, involvement in other research, etc.)

Non-specific.

2.6 How will the subjects be recruited and selected?

A list of subjects who have written to the manufacturers of myco-protein will be provided to the investigators and the subjects will be selected for ease of travel to Edinburgh.

2.7 Will any payments be made to subjects? (If so, please provide details and explanation)

Travelling expenses only.

2.8 A copy of the Patient/Subject Information Sheet MUST be attached. (See Appendix 4 for guidance notes for the Patient/Subject Information Sheet)

Where applicable, copies of all letters of introduction to patients or professionals should be attached

2.9 What investigations or interventions will be carried out on the subjects?—(Please explain in terms appropriate to an informed layman. Give details of procedures such as X-rays, cannulation, endoscopy, anaesthesia, etc.)

1. Structured clinical interview, a thorough clinical history including all aspects of general health as well as the reaction to the myco-protein-containing foods; standard clinical examination confined to the heart, respiratory system and examination of the abdomen (unless the patient has other specific symptoms which require more detailed examination)
2. In 6 people, examination of the upper gastrointestinal tract by flexible endoscopy in the baseline state, and 2 - 4 hours after ingestion of 60 grams of myco-protein.
3. Collection of pure parotid saliva and collection of whole gut lavage fluid from up to 6 myco-protein sensitive individuals in whom the clinical history suggests an immunological basis to the gastrointestinal reactions.
4. In 6 myco-protein intolerant men, or post menopausal or sterilised women; and in 6 health male volunteers, a standard radioisotope test of gastric emptying of solid and fluid phases of a test meal; in the baseline state and with myco-protein incorporated in the test meal.

2.10 What possible risks and/or discomfort to the subject can be foreseen? (State possible injuries to subjects and the probability of them occurring and specify possible discomfort, pain, limitation of activity or inconvenience likely to be incurred by subjects. Where samples are to be taken, please specify the nature and amount.)

In the subjects undergoing GI endoscopy, throat irritation and occasionally retching (the individuals will be given the option of having the procedure carried out with sedation or simply with a local anaesthetic throat spray); for the 6 subjects who may have whole gut lavage carried out, bloating, nausea and occasionally anal irritation may occur because of the volume of fluid to be ingested. Theoretical very low risk associated with the irradiation involved in the test of gastric emptying.

2.11 What measures will be taken to minimise or remove such risks or discomfort?

A dedicated Clinical Research Fellow will be involved in all of the studies and will ensure that patients are subject to as little discomfort and stress as possible.

2.12 Will the General Practitioner or consultant in overall charge of a subject be notified of the subject's participation in the study?

YES

If not, why not? (Full reasons must be given as indicated in the Guidance Notes.)

3 COMPENSATION

3.1 In the event of death, damage or lasting disability OCCURRING AS A RESULT OF NEGLIGENCE on the part of the researcher, who will accept responsibility for compensation? (A written statement from the employer of the researcher, accepting responsibility, should be provided where the researcher is NOT a contract holder with Lothian Health or an NHS Trust. Where the researcher holds a regular or honorary NHS contract with Lothian Health or an NHS Trust and is undertaking research in terms of this contract, management approval of the research will constitute acceptance of liability for negligence and MUST be obtained before research commences. In respect of research on healthy volunteers, researchers are expected to have appropriate arrangements in hand for compensation.)

UNIVED have negotiated appropriate arrangements with the company funding this research. Professor Ferguson and the Clinical Research Fellow will have membership of a medical defence union.

- 3.2 In the event of a research subject being harmed, other than by negligence, by the drugs, equipment or materials, etc. involved in the research, who will compensate subjects? (Arrangements for compensation in the event of a research subject being harmed, whether by negligence or not, will vary according to what type of body is sponsoring the research proposal but must comply with ABPI Guidelines. NHS bodies such as Trusts are NOT legally empowered to offer advance indemnity to participants in research projects.)**

As in 3.1 above.

- 3.3 If a pharmaceutical company is involved in the research, does it undertake to compensate subjects in the terms of the ABPI Guidelines? The answer to this question must include a clear statement regarding "No Fault" compensation. (A written statement from the pharmaceutical company to this effect MUST be provided. A suggested wording that WILL be acceptable is attached as Appendix 1.)**

YES/NO

REPLY - A food company is involved in the research and agrees to compensate subjects in terms analogous to the ABPI guidelines.

- 3.4 If items of medical equipment or other products under research are to be used, does the manufacturer undertake to compensate subjects?**

REPLY - Not relevant

YES/NO

4 CONSENT

- 4.1 How will informed consent be obtained from the subject, or for a minor, from the subject's parent or guardian? (The format of the sample attached as Appendix 5 should normally be used and a completed copy must be attached. If a different form is to be used it must contain all the information on the standard form and a copy must be submitted with this application.)**

In writing.

- 4.2 How much time will be allowed between giving an explanation of the research and requesting consent? (Justify if less than 24 hours)**

Subjects will be contacted at first by letter, and then a telephone discussion will be used to explain the clinical interview. Arrangements about the endoscopy, gastric emptying and gut lavage test will be made at the clinical interview, allowing a minimum of a week between giving explanation and requesting consent.

- 4.3 If the subject will be in a dependent relationship to the investigator, please state what steps will be taken to conform to "Basic Principle No. 10" of the Helsinki Declaration which states that "When obtaining informed consent for the research project, the physician should be particularly cautious if the subject is in a dependent relationship to him or her or may consent under duress. In that case the informed consent should be obtained by a physician who is not engaged in the investigation and who is completely independent of this official relationship."

Not relevant.

- 4.4 What steps will be taken to satisfy an informed layman that the subject in a study (other than a minor) who is unable or questionably able to give informed consent had indicated his/her willingness to participate? (Such as the assistance of an independent colleague, or of the next of kin, or other methods as appropriate.)

Not relevant.

- 4.5 Give the name of a doctor or other suitable person independent of the trial to whom the subject being asked to take part in the trial can refer for impartial advice and has consented to assist:

Dr Subrata Ghosh.

WHERE DRUGS ARE GIVEN IT IS RECOMMENDED THAT THE LOCAL HOSPITAL/TRUST PHARMACY MANAGER BE NOTIFIED

5 PHARMACEUTICAL OR OTHER PRODUCTS

(If no such products are to be used please go to question 6)

- 5.1 Is a pharmaceutical (or other) product(s) (e.g. appliances, prostheses, equipment for I V therapy, etc.) to be used in this study?

YES/NO

- 5.2 Name of product(s) and manufacturer: (Also specify special diets isotopes, vaccines, and state route, the doses to be used, the recommended range and duration of therapy if applicable, frequency and precautions in administration.)

Food quality myco-protein (Marlow Foods). A foodstuff used by millions of consumers throughout the world.

5.3 Mechanism of action (Describe briefly)

5.4 Usage to date (Including extent of use in man)

5.5 Toxicity and side effects (Known and anticipated, where possible avoiding technical jargon and describe the procedures and precautions in monitoring)

5.6 Withdrawal of subjects (Under what circumstances may patients be withdrawn from the study?)

Subjects may withdraw from the study at any time.

5.7 Does the pharmaceutical (or other) product possess one of the following for use in this way: REPLY - not relevant.

A Product Licence YES/NO

Product Licence No.

A Clinical Trial Certificate (CTC) YES/NO

A Clinical Trial Exemption Certificate (CTX) YES/NO

A Doctors and Dentists Exemption Certificate (DDX) YES/NO

Copies of the relevant licence or certificate **MUST** be enclosed. (In the case of the Product Licence the Licence Number will suffice.)
If the pharmaceutical (or other) product does not possess a product licence then a clear statement regarding **"No Fault"** compensation **MUST** be enclosed.

5.8 Has C.S.M. approval been given for all drugs to be used? If not, give an explanation present status of the drug(s). If the research involves the use of medicines for purposes or in ways that have not been approved by the C.S.M. and a CTC or CTX are not available, a Doctors and Dentists Exemption may be required and should be enclosed if appropriate. Please contact the Research Ethics Sub-Committee Chairman for advice.

5.9 List all other products or equipment to be used:

6 RADIATION
(If no radiation is to be used please go to question 7)

6.1 Is it intended to make any additional exposures to ionising radiations? (For example X-rays or Radionuclide investigations)

YES

6.2 If it is intended to administer radioactive substances, has approval been obtained from the Administration of Radioactive Substances Advisory Committee (ARSAC) for this particular research study? (A copy of the relevant permission should be enclosed or forwarded when available)

YES

6.3 Who is the ARSAC licence holder?

Dr Malcom Merrick.

6.4 If it is intended to use additional X-ray exposures has this been discussed with the Radiology Department?

REPLY - not relevant

YES/NO

6.5 In the case of fluoroscopy, has the person who is clinically or physically directing the X-ray exposures received adequate training in matters of radiation safety?

REPLY - not relevant

YES/NO

6.6 What is the estimated radiation dose to the patient?
(The advice of the Radiation Protection Adviser for X-rays [Mr J R Williams, Department of Medical Physics and Medical Engineering, Western General Hospital should be obtained.]

0.3 millisv per test (ie total = 0.6mSv)

7 DATA PROTECTION

7.1 Are you registered under the Data Protection Act? YES

7.2 Will all research be conducted in accordance with current codes of practice and data protection legislation?

YES

8 MANAGEMENT INFORMATION:

8.1 Does the project involve other disciplines? (e.g. General Practice Radiology, Haematology, Clinical Chemistry, Pharmacy, Nursing, etc. Please specify)

Nuclear medicine, endoscopy.

8.2 Will the project have any effect on the workload of nurses, laboratories, pharmacy, imaging, outpatient departments, etc.? Please specify. (This should be discussed with the individual departments or staff affected)

No.

8.3 Specify any resource implications for the NHS arising from this project. (Staffing, tests, pharmaceutical costs, consumables, etc.)

None. (costs of the various tests will be re-imbursed to the Trust)

8.4 By what date is it anticipated this research will have concluded?

December 1998.

9 MISCELLANEOUS

9.1 Will the project receive financial or other material support from a Government Agency, Research Council, charity, drug company, etc.? If so, specify the name and source(s) of support.

A research contract is being negotiated between the University of Edinburgh, UNIVED, and Marlow Foods.

9.2 Specify any financial or other direct benefit to investigators, their department or Institution arising from this study. Please give details: (e.g. a University Ethics Committee, if University staff or students are to be used as subjects, multi-centre trials, etc.)

No direct benefit to the investigators.

9.3 Other points that you may wish to make in justification of the proposed study:

Myco-protein based foods are proving to be highly acceptable and extremely safe, highly nutritious and relatively inexpensive new foods. It will be important to establish the mechanisms of the rare adverse reactions, so that by appropriate product labelling, individuals who may be at particular risk of developing such reactions can be forewarned.

SIGNATURE OF PRINCIPAL RESEARCHER.....

SIGNATURE OF INDEPENDENT ADVISER (As in Q 4.5).....

SIGNATURE OF SUPERVISOR (As in Q 1.6).....

NAME OF SUPERVISOR (Please print).....

ADDRESS OF SUPERVISOR.....

.....

TELEPHONE NUMBER

FAX NUMBER ,.....

DATE..... (This form is NOT valid for use after 31/12/97)

CLINICAL STUDIES OF THE MECHANISMS OF ADVERSE REACTIONS TO MYCO-PROTEIN

Background to proposed research

Myco-protein is one of several novel foods which have been developed recently. The basic constituent of myco-protein is fungal mycelia, which contains mainly protein and chitin. The material also contains egg white, which is predominantly composed of egg proteins including ovalbumin. The material is highly nutritious, of a texture similar to meat, and readily modified and flavoured to produce a wide range of food products. Myco-protein-based foods are labelled as being unsuitable for people who are allergic to mushrooms or to eggs.

The manufacturers are contacted from time-to-time by people who report that they have unpleasant reactions after eating myco-protein.

- a) Less than 5% of reports are of reactions consistent with classical allergy to a food ingredient, e.g. skin rash, swelling of the lips and mouth immediately after contact with the substance.
- b) 95% of reports are of a very consistent gastrointestinal reaction. The person vomits 2-4 hours after taking a meal containing Quorn. After vomiting has occurred, later symptoms are unusual.

Possible mechanisms of acute, allergic-type reactions

Less than 5% of reports are of reactions consistent with classical IgE-mediated allergy to a food ingredient, e.g. skin rash, swelling of the lips and mouth immediately after contact with the substance. A significant minority of the population will be sensitised, either by inhalation of fungi or by sensitisation to egg in childhood (or very, very rarely to mushrooms). Individuals already sensitised in this way could have an allergic reaction to myco-proteins even on the first exposure. It is also theoretically possible that eating myco-protein could induce food allergy to myco-protein or egg, because of physicochemical or dose effects. In this case, reactions would occur after two or more exposures.

There is abundant knowledge of egg allergy. Allergy involving fungi other than mushrooms would normally be manifest as lung disease. There are particular sub-groups (such as those with cystic fibrosis and with farmer's lung) who might in theory be at risk from IgE- (and in the case of farmer's lung, IgG-) mediated reactions to myco-protein.

Possible mechanisms of gastrointestinal reactions

95% of reports are of a gastrointestinal reaction. The person vomits 2-4 hours after taking a meal containing myco-protein. After vomiting has occurred, later symptoms are unusual. The remarkably consistent clinical pattern suggests that there is likely to be a single explanation for these reactions and that this will be a real and organic one. This could be immunological or non-immunological, and related to any of several properties of the fungus protein, the fibre-like chitin, or the egg-white proteins.

- a) IgE-mediated allergy to a component of the mycoprotein which is generated only after partial digestion, thus explaining the 2 hour delay. Were this so, reactions would

generally occur in atopic individuals known to be sensitised to other foods; or individuals with respiratory allergies involving other fungi.

b) If the mechanism of satiety relates to an effect of mycoprotein in slowing the rate of gastric emptying, an extreme variant of this, perhaps in people with co-existing mild gastrointestinal motility problems, could lead to vomiting.

c) An immune reaction (not involving IgE) to either the protein of the mycelia or egg white proteins. The time frame would suggest an IgG/IgM immune complex reaction rather than T-cell mediated. This could produce either gastritis or duodenal inflammation entirely consistent with the time frame and clinical descriptions of the reactions.

Studies to be undertaken

1. Direct clinical assessment of people who report adverse reactions to myco-protein

Structured clinical interview will be conducted with 30-40 people who report that they have had adverse reactions to myco-protein, both of the rare allergic type and the gastrointestinal reaction. This would identify co-factors relevant to likely pathogenesis including known histamine sensitivity, known atopy, egg and mushroom intolerance, respiratory diseases associated with allergy to fungi, gastroduodenal diseases including heartburn, duodenal ulcer, gastric ulcer, gastritis. It will probably be necessary to supplement information provided by the affected person, with data from patients' general practitioners, and from hospital records in some cases. I do not envisage any difficulties in obtaining this help from fellow professionals, as long as the reasons for the enquiry are clearly and courteously explained. Our experience in Scotland has been that occasionally a hospital prefers case records to be reviewed within its premises - in which case the clinical research fellow would travel to do so.

2. Studies of mechanisms of gastrointestinal reactions

2.1. In 6 people who have had severe reactions to myco-protein, upper gastrointestinal endoscopy will be performed here in Edinburgh, in the baseline state and 2-4 hours after oral feeding of myco-protein, in order to establish the presence or absence of gastritis and duodenitis.

2.2 If evidence emerges (from blood tests) to suggest that there is an immunological basis to the gastrointestinal reactions, then collection of pure parotid saliva and of whole gut lavage fluid will be performed in 6 severely affected people, with measurements of specific antibodies to myco-protein and egg antigens.

2.3 In 6 healthy male volunteers, the effects of myco-protein on gastric emptying of solid and fluid phases of test meal will be measured using standard radio-isotope techniques.

2.4 Tests of gastric emptying will also be carried out in 6 myco-protein-intolerant people, in the baseline state and with myco-protein incorporated in the test meal. Since the test involves exposure to a low dose of radiation, only men or post-menopausal or sterilised women will be asked to participate in these studies.

3. Antibodies to mycelia and egg proteins

3.1 Technical developments relating to antibodies

ELISA tests will be set up to measure antibodies of IgG, IgA and IgE classes to egg albumin, *Fusarium graminearum* and also to the fungus, *Aspergillus fumigatus*, known to have a degree of immunological cross-reactivity with *Fusarium graminearum*. Further characterisation of the proteins to which antibodies are directed will be performed by immunoblotting techniques and by competition ELISA. Antibody tests will be optimised for use in samples of blood (serum), pure parotid saliva and in intestinal secretions. Reagents for skin testing for immediate and delayed type hypersensitivity will also be developed.

3.2 Patterns of antibody in healthy individuals and in specific disease states

We have abundant amounts of material already stored, collected for other purposes, from patients with gastro-intestinal and immunological diseases. Thus, antibodies to fungal and egg antigens will be measured in serum, saliva and intestinal secretions of 20 healthy adults, 10 immunologically normal children, 10 patients with severe allergic eczema (known to have IgE, IgG and IgA antibodies to many foods), 10 patients with cystic fibrosis (known to have IgE antibodies to aspergillus), 10 patients with small bowel Crohn's disease (known to have IgG and IgA antibodies to many foods and microbial antigens including yeasts).

3.3 Patterns of antibody in people who have had clinical reactions to myco-protein

Serum and parotid saliva specimens will be tested, from up to 40 people who have had gastrointestinal reactions (e.g. those who will be attending for clinical assessment); and serum from as many as possible of those people who report allergic-type reactions (I will ask the patients' general practitioners to collect blood samples and post these to us). In addition, we will study mucosal antibodies in intestinal secretions, collected at endoscopy from the people described in section 2.2 above.

LOTHIAN RESEARCH ETHICS COMMITTEE

STANDARD CONSENT FORM

TITLE OF THE PROPOSED RESEARCH:

Clinical studies of the Mechanisms of Adverse Reactions to Myco-Protein

NAME OF INVESTIGATOR:

Professor Anne Ferguson

ADDRESS:

Gastrointestinal Unit, Western General Hospital, Crewe Road, Edinburgh EH4 2XU

TELEPHONE:

0131 537 1731

FURTHER INFORMATION IS AVAILABLE FROM: (A person who is not involved in the study)

Dr Subrata Ghosh

LIST ANY DRUGS TO BE GIVEN IN THE STUDY EXPLAINING THEIR ACTION:

none

LIST ANY PROCEDURES REQUIRED IN ADDITION TO THE STANDARD PROCEDURES:

For most patients, only a general clinical assessment will be carried out.

In selected patients (the study doctor will discuss with you if we are planning to invite you to be one of these selected patients) depending on the nature of the clinical history of reactions, one or more of the following tests may be done.

1. Upper gastrointestinal endoscopy on two occasions.
2. Whole gut lavage test on one occasion.
3. Test of gastric emptying on two occasions.

- I agree to participate/to the patient/subject participating* in this study.
 - I have read this consent form and Patient/Subject Information Sheet and had the opportunity to ask questions about them.
 - I agree for notice to be sent to my/the patient's/subject's* General Practitioner about my/their* participation in this study.
 - I agree to the provision of any clinically significant information to my/the patient's/subject's General Practitioner.
 - I understand that I am/the patient/subject is* under no obligation to take part in this study and that a decision not to participate will not alter the treatment that I/the patient/subject* would normally receive.
-
- I understand that I have/the patient/subject has* the right to withdraw from this study at any stage and that to do so will not affect my/their* treatment.
 - I understand that this is non-therapeutic research from which I/the patient/subject* cannot expect to derive any benefit.*

Signature of Patient/Subject/Parent or Guardian*

Name of Patient/Subject:

Signature of Investigator:

Date:

*** Delete as appropriate**

Four copies to be made

Top copy to be retained by Investigator

Second copy to be retained by patient/subject

Third copy to be sent to patient's/subject's General Practitioner

An additional copy to be filed in any relevant hospital case notes

GUT LAVAGE TEST FOR RESEARCH STUDIES

Information for patients and volunteers.

Antibodies are the molecules which neutralise and kill infectious agents such as bacteria and viruses. Certain types of antibodies can also cause allergic diseases and diarrhoea.

Tests on blood give very little information on gut immunity because the relevant antibodies are present in digestive juices from the stomach and lower down in the bowel. Our recent research has led to the discovery of the gut lavage test, a simple and safe way for us to obtain digestive juices from the intestine, so that measurements of intestinal antibodies can be performed.

What the gut lavage test would mean for you is as follows:

(The test is normally performed in a quiet area of the Western General Hospital, staffed by specially trained nurses).

You can eat and drink normally until midnight.

The next morning, you will be given a glass of bland fluid to drink, every 10-15 minutes for about two hours.

The fluid passes along the bowel (like water along a hosepipe). After 1 - 1 1/2 hours your bowels will move normally a couple of times. Then you will pass clear fluid from the bowl. This clear fluid will be collected by a nurse or doctor.

The test is then complete and you can eat and drink normally. You should expect to have several more watery bowel motions in the course of the day.

While you are having the gut lavage test, a doctor will record some simple details of your present and past health. A blood sample will also be taken for reference purposes.

Thank you for offering to help with our research. You should not find the test distressing, but please note that if you do you are completely free to stop the test at any time.

INFORMATION FOR PATIENTS RE GASTROINTESTINAL ENDOSCOPY

Thank you for agreeing to have a gastrointestinal endoscopy test on two occasions, as part of our research programme on clinical reactions to myco-protein.

The endoscopy test is very straight forward. We will ask you to fast for about 4 hours prior to the test. There is the option of having the test carried out quickly with a local anaesthetic spray to your throat and no other sedative, or alternatively a simple injection of a tranquilliser into a vein which makes you sleepy for 1 - 2 hours, you have a natural sleep after the procedure and normally recall very little of it.

At endoscopy a slim black tube will be passed by the doctor through your throat into the stomach and duodenum, and if there are any patches which look inflamed, small biopsies, about the size of a rice grain, will be taken through the instrument.

You may feel irritation at the back of your throat, and patients occasionally are nauseated and retch a little.

We will ask you to have a second endoscopy, two to four hours after taking a meal of myco-protein which we will provide. As we will explain to you when you attend for clinical assessment, the reason for this is to see if the myco-protein produces irritation or inflammation of the lining of the stomach. Once again, small biopsies will be taken if there are any apparently inflamed areas in the stomach.

Note that you are completely free to decide not to continue with the research study and the endoscopy, at any time.

One of the GI research team doctors will be present when you are having the endoscopy tests.

INFORMATION FOR PATIENTS AND VOLUNTEERS ABOUT THE TEST OF GASTRIC EMPTYING

Thank you for agreeing to have a test of gastric emptying as part of the research project on reactions to myco-proteins.

The procedure is straight forward. You will be asked to come to the hospital having fasted from midnight the evening before. On one occasion, you will have the standard test; and on a second occasion you will have the standard test to which a small amount of myco-protein has been added.

The standard test involves taking some scrambled eggs together with a drink. There is a tiny trace of radio activity incorporated in these materials (equivalent to one or two days background exposure in the UK).

You will then have the radio activity in your stomach area measured by a simple machine. All we will ask you to do is sit quietly and have the radio activity pattern of your abdomen checked every 15 minutes or so for up to 3 hours.

If at any time you wish to discontinue the test then you are completely free to do so. One of the GI Research team will be present when you are having the gastric emptying test, in case you have any concerns or problems during the procedure.

1702/97/4/109



OUR REF:

YOUR REF:

05 August 1997

Professor A Ferguson
GI Unit
Western General Hospital
Crewe Road
EH4 2XU

Dear Professor Ferguson

Research protocol 1702/97/4/109: Clinical studies of the mechanisms of adverse reactions to myco-protein.

Thank you for submitting the above protocol for ethical approval. The Medicine and Clinical Oncology Research Ethics Sub-Committee will consider this protocol at its next meeting which will be held on Wednesday 13 August 1997. I will notify you of the outcome of this consideration as soon as possible.

Under the terms of the Scottish Office Home and Health Department Guidelines on Local Research Ethics Committees a copy of your request has been sent to the NHS body under the auspices of which the research is intended to take place. It is that NHS body which has the responsibility of deciding whether or not the research should go ahead taking account of the advice of the Research Ethics Sub-Committee.

Yours sincerely

Linda Semple
Secretary
Medicine and Clinical Oncology
Research Ethics Sub-Committee

Please quote the above reference on all correspondence



3 September 1997

Professor A Ferguson
Gastrointestinal Unit
Western General Hospital
Crewe Road
Edinburgh EH4 2XU.

Dear Professor Ferguson,

Request for Ethical Approval—1702/97/4/109 : Clinical studies of the Mechanisms of adverse reactions to myco-protein

Thank you for submitting the above protocol for ethical approval. The Medicine/Clinical Oncology Research Ethics Sub-Committee has discussed this protocol but has deferred making a decision and continued consideration of the application for the following reasons:

- *a fuller Patient Information Sheet is required giving more details about the study and including the name/address etc of the Independent Adviser*
- *clarification of whether the subjects will have all the tests or only one*
- *the significance of having groups of 6 people*
- *an indication of the risk of potential harm to subjects who have a protein allergy*

The next meeting of the Sub-Committee will be held on 1 October 1997. It would be appreciated if the required amendments could be available prior to that date.

Should you have any queries regarding the above, please contact myself on extension 9050.

Yours sincerely,

Linda Semple
Secretary
Medicine/Clinical Oncology
Research Ethics Sub-Committee